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The circulating proteinase inhibitor alpha-1 antitrypsin regulates neutrophil degranulation and autoimmunity

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One sentence summary: Alpha-1 antitrypsin regulates neutrophil driven autoimmunity

Abstract

Pathological inflammation and autoimmune disease frequently involves elevated neutrophil activity in the absence of infectious agents. Tumour necrosis factor- α (TNF- α) contributes to many of the problems associated with autoimmune diseases. In this study we investigated the ability of serum alpha-1 antitrypsin (AAT) to control TNF- α biosynthesis and signaling in neutrophils and assessed whether AAT deficiency (AATD) is a TNF- α related disease. *In vitro* studies demonstrate that serum AAT coordinates TNF- α intracellular signaling and neutrophil degranulation of tertiary and secondary granules via modulation of ligand-receptor interactions. AATD patients homozygous for the Z-allele were characterised by increased activation of the TNF- α system, as demonstrated by increased membrane TNF- α levels and increased plasma concentrations of TNF receptor 1 (TNF R1) and neutrophil released secondary and tertiary granule proteins. The incidence of autoantibodies directed against degranulated lactoferrin, and surface protein accessible to these antibodies was increased in ZZ-AATD, leading to an enhanced rate of neutrophil reactive oxygen species production. Treatment of ZZ-AATD individuals with AAT augmentation therapy results in decreased membrane TNF- α expression and plasma levels of granule antigenic proteins and IgG-class autoantibodies. These results provide a mechanism by which AAT augmentation therapy impacts on TNF- α signaling in the circulating neutrophil, indicating promising potential of this therapy for other TNF- α related diseases.

Introduction

Alpha-1 antitrypsin deficiency (AATD) is a hereditary disorder characterised by low circulating levels of the key antiprotease alpha-1 antitrypsin (AAT). AATD is associated with the development of chronic obstructive pulmonary disease (COPD) often by the 3rd or 4th decade, liver disease and in rare cases skin panniculitis. The most common SERPINA1 mutation associated with AATD is the Z mutation and the vast majority of AATD individuals diagnosed with COPD are homozygous for the Z allele. Current treatment of lung disease associated with AATD is AAT augmentation therapy, whereby patients receive weekly infusions of plasma purified AAT. Intravenous administration of AAT has been shown to be safe and well tolerated (1, 2) and results in increased levels of AAT in bronchoalveolar lavage fluid of individuals with AATD (3). Observational studies on the clinical efficiency of AAT augmentation therapy include a trend towards a slower rate of FEV₁ decline (4) and a reduction in the incidence of lung infections (5). The EXACTLE study published in 2009 demonstrated the efficacy of AAT augmentation therapy in preventing the loss of lung tissue as measured by computed tomography scan lung density (6). However, despite the observed clinical effects of AAT further proof of the effectiveness and mechanism of action of augmentation therapy is required (7).

The notion of AAT as an anti-inflammatory mediator is gaining acceptance (8-12). AAT has been shown to possess substantial anti-inflammatory properties independent of its antiprotease activity affecting a number of cell types, including pancreatic islet β -cells (13), B-cells (14), mast cells (15) and macrophages (16, 17) and has been implicated in cellular processes as diverse as endothelial cell apoptosis (11), neutrophil chemotaxis (18) and fibroblast mediated cytokine expression (19). Indeed, it is now accepted that AAT has a number of functions beyond protease

inhibition highlighting the use of AAT augmentation therapy for a range of diseases other than AATD. In this regard, it was discovered that AAT reduced inflammation in pancreatic islet cells which lead to multiple studies investigating the potential role of AAT in treatment of diabetes (20-24). Other immune-mediated disease models in which the effect of AAT has been explored include arthritis (14, 19), Crohn's disease and acute myocardial infarction (25). AAT has also been evaluated as a human immunodeficiency virus type 1 (HIV) antagonist and was shown to prevent viral replication (26-28). Moreover, the therapeutic effect of AAT investigated in airway diseases, not associated with AATD, includes bronchiectasis/COPD and cystic fibrosis (2, 17, 29-31).

TNF- α , a potent pro-inflammatory cytokine that operates at an early stage in COPD (32, 33) and in other chronic illnesses including cystic fibrosis (34, 35) and asthma (36), may also contribute in the development of disease associated with AATD. Studies supporting this hypothesis have demonstrated a reduction in TNF- α levels by AAT in a murine model of emphysema (37) and a positive correlation between AAT inactivation and TNF- α concentrations has been established in patients with rheumatoid arthritis (RA) (38) and cystic fibrosis (39). The consequence of elevated levels of TNF- α is evident as TNF- α blockade has transformed treatment of several autoimmune diseases including RA, psoriasis, ankylosing spondylitis and inflammatory bowel disease (40). Several small studies have suggested that carriage of the Z-allele in AATD is associated with development of autoimmunity and antineutrophil cytoplasmic antibodies (ANCA) (41, 42), causing aberrant neutrophil activation and ANCA-associated vasculitis (43). Therefore, with the aim of clarifying the role of TNF- α in AATD related disease, we investigated the impact of AATD on TNF- α neutrophil activation. This study has identified a pathogenic mechanism

associated with AATD and illustrates the anti-inflammatory efficiency of AAT augmentation therapy by modulating activation of the TNF- α system and autoimmune response.

Results

Elevated plasma TNF- α and neutrophil secondary and tertiary granule proteins in ZZ-AATD.

To determine the role of TNF- α and its potential effect on neutrophil function *in vivo*, cells and plasma samples were obtained from healthy controls (N=10, MM) and non-obstructed ZZ-AATD patients (N=10, Table 1). Quantification of TNF- α by ELISA revealed that ZZ-AATD neutrophils cultured for 6 h produced four times more soluble TNF- α than that measured in supernatants of MM cells (36.86 ± 7.75 pg/ml and 9.10 ± 5.44 pg/ml respectively, $P=0.03$) (Fig. 1A). Analysis of TNF- α plasma levels however revealed no significant difference between MM controls and non-obstructed ZZ-AATD patients (3.5 ± 0.26 pg/ml and 3.9 ± 0.43 pg/ml, respectively, $P=0.4$) (Fig. 1B). This disparity between *in vitro* and *in vivo* findings can be explained by the fact that TNF- α is not readily detected in biological samples (44), possess a relatively short half-life (45) and is rapidly excreted from the body (46). Thus, to further evaluate TNF- α levels *in vivo*, flow cytometry analysis of the relative mean fluorescence intensity (MFI) of TNF- α on the membrane of peripheral blood neutrophils was performed (Fig. 1C). Results revealed that ZZ-AATD cells had a 1.5-fold increased membrane expression of TNF- α when compared to MM neutrophils (1.59 ± 0.19 and 1.07 ± 0.06 MFI respectively, $P=0.04$). Moreover, it has previously been shown that soluble plasma TNF R1 concentrations can act as a surrogate marker for TNF- α levels (47-49). Thus quantification of TNF R1 levels in control and patient plasma was performed (Fig. 1D), with results revealing 36% less TNF R1 in healthy control MM plasma when compared to ZZ-AATD patient samples (580.6 ± 41.93 pg/ml and 906.9 ± 75.43 pg/ml respectively, $P=0.0008$).

As neutrophil exposure to TNF- α has been shown to cause degranulation of tertiary and secondary but not primary granules (50), levels of specific components of the tertiary and secondary granules were quantified in plasma samples. Matrix metalloproteinase 9 (MMP-9) a marker of tertiary granule release was statistically higher in non-obstructed ZZ-AATD plasma compared to control MM samples ($P=0.0002$) (Fig. 1E). Moreover, results indicated significantly increased degranulation of secondary granules by AATD neutrophils *in vivo*, as a 3- and 1.8-fold increase was observed in levels of human cathelicidin antimicrobial protein 18 (hCAP-18) (Fig. 1F) and lactoferrin (Fig. 1G) in ZZ-AATD plasma compared to control samples ($P=0.02$ and $P=0.03$, respectively). Overall these results demonstrate that TNF- α induced inflammation plays a central role in ZZ-AATD and impacts upon neutrophil degranulation processes.

Dysregulated degranulation pattern of neutrophils from ZZ-AATD patients.

As greater levels of tertiary and secondary granule components were detected in plasma of non-obstructed ZZ-AATD patients, a comparison of the rate of neutrophil degranulation between MM control donors and individuals with the ZZ phenotype was performed. Results demonstrated that unstimulated ZZ-AATD neutrophils released a significantly higher level of granule proteins (Fig. 2A-2C). In comparison to the MM cell, at the 5 min time point the ZZ neutrophil released a 2-, 8- and 9-fold increase in the level of MMP-9, hCAP-18 and lactoferrin, respectively. After a longer period of incubation (20 min) there was a 2-, 2.5- and 3-fold increase in the level of secreted MMP-9, hCAP-18 and lactoferrin respectively, by ZZ-AATD neutrophils compared to MM cells ($P=0.04$, $P=0.03$ and $P=0.02$, respectively) (Fig. 2A-2C).

Results also revealed that ZZ-AATD neutrophils exhibited an increased degranulation response to TNF- α (10ng/2x10⁷/ml) compared to control cells (Fig. 2D-2F). A significant difference was observed at all individual time points measured from 5 min onwards, with at least a 2-fold increase in ZZ-AATD neutrophil degranulation of MMP-9, hCAP-18 and lactoferrin compared to MM cells after 20 min (Fig. 2D-F; P=0.03, P=0.01 and P=0.02 respectively). Control experiments confirmed that freshly isolated MM and ZZ-AATD cells contained similar levels of MMP-9, hCAP-18 and lactoferrin (fig. S1) and that the concentration of TNF- α employed in degranulation experiments for 20 min did not induce apoptosis or cell death as verified by annexin V and propidium iodide staining of neutrophils (fig. S2). Collectively, these results demonstrate that ZZ-AATD neutrophils release increased levels of secondary and tertiary granules both under unstimulated and TNF- α stimulated conditions, possibly indicating that the circulating ZZ-AATD cell is in a primed state.

AAT activity regulates TNF- α induced neutrophil degranulation.

To investigate whether the observed increased exocytosis of tertiary and secondary granules by ZZ-AATD neutrophils was a result of low AAT levels, the effect of exogenous AAT employed at physiological levels (27.5 μ M) on TNF- α induced MM and ZZ AATD neutrophil degranulation was examined (Fig. 3). As illustrated in Fig. 3A, C and E, the increased degranulation of MMP-9, hCAP-18 and lactoferrin induced by TNF- α in MM cells was significantly inhibited by AAT, with a 2-fold decrease observed for all proteins at the 20 min time point (P=0.04, P=0.03 and P=0.04, respectively). The effect of a second serum derived serpin, antithrombin III, on TNF- α induced neutrophil degranulation was also examined. After 20 min, a

physiological concentration of antithrombin III (4 μ M) was shown to have no effect on TNF- α induced degranulation by MM cells (Fig. 3A, 3C and 3E).

The inhibitory effect of AAT on TNF- α induced degranulation was also examined on neutrophils from non-obstructed ZZ-AATD individuals (Fig. 3B, 3D and 3F). The results demonstrated that after 20 min, there was a trend for reduced levels of MMP-9, hCAP-18 and lactoferrin release by ZZ-AATD cells in response to TNF- α in the presence of physiological levels of AAT when compared to TNF- α alone, although this reduction did not reach significance. Evaluation of the impact of AAT on TNF- α signaling and its ability to stimulate MAP kinase p38 phosphorylation, a key step in the neutrophil degranulation process (51), revealed that AAT reduced p38 phosphorylation in a dose dependent manner with an IC₅₀ of 29.15 μ M (fig. S3). Increasing the concentration of AAT to levels observed during inflammation (55-110 μ M) (52) caused a further decline in p38 phosphorylation (fig. S3A). The study also demonstrated an AAT inhibitory dose-response on TNF- α induced degranulation of MMP-9, hCAP-18 and lactoferrin (fig. S3B, S3C and S3D, respectively). Overall this set of experimental results confirms the ability of plasma AAT to regulate TNF- α induced neutrophil degranulation.

Excessive neutrophil degranulation gives rise to the development of autoantibodies in AATD.

As a consequence of increased neutrophil degranulation and high plasma levels of secondary and tertiary granule proteins in ZZ-AATD, the potential for the development of autoantibodies in AATD was evaluated. A comparison of anti-MMP-

9, anti-hCAP-18 and anti-lactoferrin IgG-class autoantibodies in plasma from patients with ZZ-AATD and healthy controls was performed (Table 2, N=30). Overall the level of anti-lactoferrin IgG antibodies were significantly higher in the ZZ-AATD group when compared to control donors ($P=0.001$), but there was no significant difference in the level of anti-MMP-9 or anti-hCAP-18 IgG autoantibodies (Fig. 4A-4C). By employing a predetermined threshold level for positivity previously described as 3 standard deviations above the mean of MM healthy controls (53), eight of the 30 ZZ-AATD individuals tested proved positive for autoantibodies against lactoferrin, higher than that observed for the other granule proteins (Fig. 4A-4C). Moreover, the specificity of plasma purified anti-lactoferrin IgG antibody was confirmed in a competitive assay by Western blot analysis (Fig. 4D). Results demonstrated that exogenous lactoferrin protein (5 μ g) negatively impacted on the ability of patient purified anti-lactoferrin antibody to bind to membrane immobilised lactoferrin, resulting in a 5 fold decrease in the signal obtained ($P=0.008$) (Fig. 4D). Further analysis revealed no significant increase in IgM titre against all three granule proteins in ZZ-AATD compared to MM control samples, with 1/30, 1/30 and 5/30 patients positive for IgM autoantibodies against MMP-9, hCAP-18 and lactoferrin, respectively (fig. S4).

Next, the possibility that autoantibody cross-linking of antigens bound to the outer cell surface could trigger signal transduction and neutrophil activation was examined. As IgG class autoantibodies directed to lactoferrin of neutrophils were the most prevalent in ZZ-AATD, we examined anti-lactoferrin antibody-mediated ROS production as a read-out system for cell activation. It was first necessary to confirm membrane surface expression of lactoferrin thus enabling interaction between antibody and antigen. Western blot analysis of isolated neutrophil plasma membranes

demonstrated significantly higher levels of lactoferrin on ZZ-AATD cells compared to healthy control samples ($P < 0.0001$) (Fig. 4E). This was further validated by flow cytometry analysis of non-permeabilized cells confirming higher expression of lactoferrin on the ZZ-AATD neutrophil (1.90 ± 0.26 MFI) when compared to the MM cell (1.12 ± 0.11 MFI, $P = 0.02$) (Fig. 4F). Furthermore, incubation of TNF- α -primed MM neutrophils with commercially available anti-lactoferrin IgG resulted in superoxide (O_2^-) production similar to TNF- α -primed cells activated with fMLP, albeit at a slower rate (Fig. 4G). Isotype control antibody or TNF- α alone had no stimulatory effect. In addition, incubation of ZZ-AATD neutrophils with anti-lactoferrin antibody for 30 min caused a robust increase in extracellular O_2^- production, and in comparison MM cells released significantly less (55% lower levels, $P = 0.004$) (Fig. 4H). Whilst additional TNF- α -priming of ZZ-AATD neutrophils *in vitro* had little effect on the level of O_2^- produced, TNF- α -priming of MM cells significantly increased O_2^- production in response to anti-lactoferrin IgG, lending further support to the primed state of the ZZ-AATD circulating cell (Fig. 4H). In addition, experiments exploring the effect of anti-lactoferrin autoantibodies purified from ZZ-AATD plasma revealed that purified anti-lactoferrin autoantibodies mediated activation of cells. Incubation of TNF- α -primed MM neutrophils with patient plasma purified anti-lactoferrin autoantibodies resulted in O_2^- production similar to primed cells activated with fMLP (Fig. 4I). From this set of experiments we conclude that autoantibodies directed against lactoferrin circulating in the plasma of ZZ-AATD patients, can target antigen bound to the cell surface and trigger neutrophil ROS production.

AAT regulates TNF- α signaling by modulating TNF- α receptor interaction.

Results so far have demonstrated the ability of AAT to modulate neutrophil signaling in response to exogenous TNF- α . In endothelial cells, studies have shown that TNF- α can regulate its own gene expression (9). Thus in the present study the ability of AAT to affect TNF- α self-regulated gene and protein expression in the neutrophil like human promyelocytic HL-60 cell line was examined. HL-60 cells (10^7 /ml) were incubated with TNF- α in the presence or absence of physiological concentrations of AAT (27.5 μ M) for 6 h. Results demonstrated that AAT down-regulated TNF- α signaling and functioned to significantly reduce TNF- α gene expression in HL-60 cells ($P=0.03$) (Fig. 5A). The mechanisms of inhibition were shown to involve the ability of AAT to prevent TNF- α induced activation of NF- κ B, as demonstrated by reduced I κ B α degradation (Fig. 5B). In support of these results, we observed significantly increased NF- κ B activation in non-obstructed ZZ-AATD neutrophils when compared to healthy control cells (Fig. 5C) ($P=0.04$). Collectively, these results indicate that ZZ-AATD neutrophils have increased NF- κ B activation, further suggesting that AATD is associated with a pro-inflammatory phenotype in circulating neutrophils.

Ensuing experiments investigated the possible mechanism by which AAT inhibits TNF- α induced signaling, degranulation and gene upregulation. The ability of AAT to interact with TNF- α was examined as previous studies have demonstrated that AAT can bind a range of inflammatory mediators (18, 54-56). However, results of a Biacore based assay whereby TNF- α was coupled to a CM-5 chip demonstrated that no binding event occurred between AAT and TNF- α (Fig. 5D). As a positive control a monoclonal antibody against TNF- α was used with a positive binding event

of 60 RU detected (Fig. 5D). Ensuing experiments determined whether AAT impacted on TNF- α binding to its two receptors, TNF R1 and TNF R2. The exposure of TNF R1 and TNF R2 coated surfaces to TNF- α (10ng) in the presence or absence of AAT (27.5 μ M) revealed that AAT significantly reduced TNF- α receptor engagement. In this regard, AAT significantly reduced TNF- α binding to TNF R1 by 35% (P=0.03) (Fig. 5E), with a similar result observed for TNF R2 (50% reduction; P=0.02) (Fig. 5F). As a positive control, a TNF R1 Fc chimera (TNF- α blocker) was utilised, resulting in a 80% reduction in the level of TNF- α binding to TNF R1 and R2 (P<0.0001) (Fig. 5E and 5F).

A previous study has established that AAT can interact with the neutrophil through receptors exposed on the membrane surface (18). Thus ensuing experiments examined whether AAT may block TNF- α receptor engagement by binding TNF R1 and TNF R2. Polybeads coated with TNF R1, TNF R2 or the IgG receptor Fc γ RIIA (the latter acting as a negative control shown previously not to bind to AAT (18)), were incubated with AAT followed by a FITC labeled antibody against AAT to confirm AAT binding by flow cytometry (Fig. 5G). Results demonstrated a significant increase in AAT binding to both TNF R1 (2.44 ± 0.38 MFI) and TNF R2 (2.43 ± 0.48 MFI) when compared to unlabelled (0.88 ± 0.08 MFI, P=0.01 and P=0.04 respectively) or Fc γ RIIA control beads (1.35 ± 0.16 MFI, P=0.03 and P=0.02 respectively). There was no difference in the level of AAT binding to unlabeled or Fc γ RIIA control beads.

The ability of TNF- α to bind to its receptors on the neutrophil membrane *in vitro* in the presence of AAT was next evaluated. By flow cytometry the ability of exogenous TNF- α (10ng) to bind neutrophil membranes in the absence or presence of

AAT (27.5 μ M) was examined utilising an antibody that could detect receptor bound TNF- α . Results revealed that cells pre-incubated with physiological levels of AAT (27.5 μ M) and then exposed to TNF- α exhibited a 55% reduction in the level of bound TNF- α compared to cells exposed to TNF- α only (1.20 ± 0.08 MFI and 2.7 ± 0.03 MFI, respectively, $P < 0.0001$) (Fig. 5H). Collectively, these results indicate that TNF- α signaling can be regulated by physiological concentrations of AAT and that this regulation occurs at the membrane level, negating TNF- α receptor interaction.

An evaluation of the effects of long term administration of AAT augmentation therapy in ZZ-AATD patients.

Thus far results have demonstrated the ability of AAT to modulate TNF- α signaling and the downstream effects on neutrophil function *in vitro*. Next the impact of AAT on TNF- α signaling *in vivo* was examined by investigating the impact of AAT augmentation therapy. Samples were isolated from ZZ-AATD patients possessing an FEV₁ of 30-70% predicted and who were on augmentation therapy for over 4 years (N=3), ZZ-AATD patients who had never received augmentation therapy (N=3, FEV₁ 30-70% predicted) and healthy controls. Flow cytometry analysis of AAT on neutrophil membranes illustrated a 2-fold increase in ZZ-AATD patients currently receiving augmentation therapy when compared to ZZ-AATD patients who have never received AAT infusions (1.09 ± 0.18 and 0.56 ± 0.03 MFI respectively, $P = 0.04$) (Fig. 6A). Subsequent analysis of membrane TNF- α levels revealed that augmentation therapy caused a 1.7-fold decrease in TNF- α expression on ZZ-AATD neutrophil membranes when compared to cells isolated from ZZ-AATD patients who had never received AAT treatment (0.95 ± 0.15 and 1.62 ± 0.10 MFI respectively,

P=0.01) (Fig. 6B). Moreover, the membrane expression levels of TNF- α were similar between ZZ-AATD patient cells on augmentation therapy and MM control neutrophil membranes (1.00 ± 0.16 MFI) (Fig. 6B). In addition, the level of soluble TNF R1 detected in plasma samples was decreased by approximately 30% in ZZ-AATD patients receiving augmentation therapy when compared to patients not receiving treatment (827.4 ± 92.37 pg/ml and 1258 ± 124.9 pg/ml respectively, P=0.03) (Fig. 6C). The levels recorded in the augmentation therapy group were not statistically different when compared to the level of soluble TNF R1 in MM controls. Analysis of the soluble TNF R2 receptor levels in plasma revealed no significant difference between MM, ZZ-AATD patients receiving AAT augmentation therapy and ZZ-AATD patient group who had never received AAT infusions (fig. S5). This is in line with a previous study indicating that levels of soluble TNF R1, but not TNF R2, are elevated in COPD (57).

Finally, as data from this study has demonstrated the presence of circulating levels of autoantibodies against lactoferrin in ZZ-AATD patients (Fig. 4C), we evaluated autoantibody titre levels in plasma from ZZ-AATD individuals (N=4) prior to starting augmentation therapy and 4 years post treatment. As illustrated in Fig. 6D, results demonstrated that the titre of anti-lactoferrin autoantibodies decreased by 30% after four years of receiving AAT infusions (P=0.04), thus indicating the benefit of long term therapy in suppressing autoimmunity.

The impact of weekly AAT augmentation therapy infusions upon neutrophil degranulation.

Weekly infusions of AAT augmentation therapy increase the serum levels of AAT in AATD patients, but eventually these levels rapidly decline from normal to near or below that which is considered the “protective threshold” of 11 μ M prior to the patient’s next infusion (3). This led us to evaluate the impact of the pharmacokinetics of weekly AAT augmentation therapy infusions (60mg/kg of patient body weight) on TNF- α and neutrophil degranulation markers. Plasma and neutrophils were isolated from ZZ-AATD patients who were receiving weekly AAT augmentation therapy with samples collected on day 2 and day 7 post infusion. The impact of AAT augmentation therapy resulted in significantly increased levels of AAT on day 2 compared to day 7 post treatment (30.2 μ M and 7.59 μ M respectively, $P=0.004$) (Fig. 7A). Results of flow cytometry analysis revealed that ZZ neutrophils illustrated significantly reduced levels of membrane associated TNF- α on day 2 post treatment (0.77 ± 0.12 MFI) when compared to day 7 cells (1.11 ± 0.13 MFI, $P=0.0004$) (Fig. 7B). To investigate whether AAT augmentation therapy corrected the dysregulated degranulation pattern of ZZ-AATD neutrophils, the CD66b membrane expression profile of ZZ-AATD neutrophils was assessed by flow cytometry analysis (Fig. 7C). CD66b is a membrane receptor that is exclusively present on secondary and tertiary granules (58) and upon degranulation becomes expressed on the cell surface. When compared to day 2 neutrophils (0.74 ± 0.12 MFI), day 7 cells demonstrated a significant increase in CD66b on neutrophil membranes (1.02 ± 0.08 MFI, $P=0.03$), indicative of increased levels of degranulation. This latter result was supported by lower concentrations of degranulated secondary and tertiary granule proteins in plasma samples on day 2 when compared to day 7 post treatment. Zymographic analyses revealed an approximate 25% increase in circulating plasma levels of MMP-9 on day 7 post treatment when compared to day 2 ($P=0.01$) (Fig. 7D). Likewise, there was a

significant increase in the plasma levels of hCAP-18 and lactoferrin on day 7 post augmentation therapy when compared to day 2 post treatment ($P=0.03$ and $P=0.04$ respectively) (Fig. 7E and 7F). Collectively, these results illustrate the effect of weekly AAT infusions on TNF- α membrane expression and the degranulation activity of circulating neutrophils *in vivo*, both of which are clearly related to the levels of AAT in plasma.

Discussion

Classically, AAT has been considered an important modulator of lung disease through inhibition of serine proteases and restoration of the antiprotease / protease balance in the airways (31, 59). More recently however, reports have highlighted anti-inflammatory attributes of AAT including inhibition of cell apoptosis (60, 61). In the present study we demonstrate that AAT plays a direct role in modulating TNF- α signaling in neutrophils and functions to decrease the degranulation process of circulating cells. We have elucidated a role for AAT and uncovered the capacity of this serum protein to modulate the degranulation response of TNFR1 and TNFR2 receptor signaling by interrupting ligand-receptor interaction. In AATD, this regulatory mechanism is considerably perturbed and an association between low plasma levels of AAT and high levels of exocytosed granule proteins including lactoferrin, hCAP-18 and MMP-9 was observed, characteristic of an *in vivo* hyper-responsive state of AATD neutrophils. Consequently, patients with AATD can potentially develop agonistic autoantibodies against granule proteins including lactoferrin, which in turn can cause neutrophil activation and ROS production (Fig. 8).

Circulatory levels of soluble TNF R1 have been shown to be elevated in inflammatory lung conditions including COPD (62, 63), and TNF R1 is thought to be a more reliable marker of activation of the TNF- α system due to its high concentration and stability (44, 62). Within this study, we observed a significant increase in plasma levels of soluble TNF R1 in ZZ-AATD patients compared to MM individuals. Furthermore, a 1.5-fold increase in TNF- α membrane expression was detected in ZZ-AATD neutrophils when compared to healthy controls, a level similar

to that documented on neutrophils of individuals with RA (64). In this study we also show that ZZ-AATD neutrophils secrete significantly higher levels of TNF- α . These results indicated that TNF- α could play a key role in the progression of AATD related disease and raised the possibility that AAT could modulate TNF- α bioactivity. In this regard, TNF- α is a potent inducer of the release of neutrophil secondary and tertiary granules (65). At baseline and also in response to TNF- α , neutrophil degranulation capacity was significantly higher in ZZ-AATD patients, with elevated levels of secondary (hCAP-18 and lactoferrin) and tertiary (MMP-9) granule proteins released compared to healthy MM control cells. TNF- α does not cause release of primary granules (50) and this was further confirmed within this study as only hCAP-18 precursor protein (18kDa) was detected by Western blotting due to the requirement of proteinase-3 for its activation (66), a serine protease component of primary granules. The findings of enhanced TNF- α -induced degranulation by ZZ-AATD neutrophils are in keeping with a pivotal role for AAT in modulation of TNF- α signaling and in support of this theory, physiological concentrations of AAT (27.5 μ M) exhibited significant inhibition of degranulation by MM control cells in response to TNF- α . This inhibition of TNF- α signaling was specific to AAT as another serpin member and acute phase protein, antithrombin III, had no effect on TNF- α induced neutrophil degranulation. In line with these results, a previous study by Mansell *et al.*, (2001) demonstrated the inability of antithrombin III to regulate TNF- α signaling in a monocytic cell line (67). Moreover, experiments assessing the effect of AAT on ZZ-AATD cells revealed that AAT had a lesser inhibitory effect on TNF- α induced degranulation compared to MM neutrophils. This latter observation is possibly due to the primed state of the ZZ-cell, as evident by the increased level of membrane TNF- α ,

which has previously been shown associated with priming (68). Thus, by corollary results of our study also indicate that AAT is incapable of de-priming the cell, a phenomenon that has been previously documented to occur in neutrophils (69, 70).

One attribute associated with TNF- α signaling is its ability to self-regulate its own gene expression (71), as well as the expression of other inflammatory mediators through the NF κ B pathway (72, 73). Similar to the effect observed in endothelial cells (9), our *in vitro* studies found that AAT down regulated TNF- α gene expression in response to exogenous TNF- α and further revealed that AAT caused a blockade of I κ B α degradation. Moreover, Western blot analysis of whole cell lysates of isolated peripheral blood neutrophils indicated lower levels of I κ B α in ZZ-AATD cells when compared to MM controls suggesting elevated NF κ B activation in AATD. This result is comparable to that observed in neutrophils isolated from patients with RA, which at baseline exhibited higher NF κ B activation when compared to the relative control group (64).

The observed excessive neutrophil degranulation by ZZ-AATD cells prompted us to question the consequence and possible impact on disease progression. A number of studies have indicated that the Z allele is increased in ANCA-associated vasculitis, with 5-27% of individuals with granulomatosis with polyangiitis carrying the Z allele (74-77). Based on the fact that lactoferrin is a highly abundant neutrophil protein (78, 79) and also as elevated levels of this iron-binding protein were detected in ZZ-AATD plasma, we evaluated an association with the development of autoantibodies. Results revealed that of 30 ZZ-AATD patients, 27 % were positive for anti-lactoferrin IgG while only 17 % were positive for anti-lactoferrin IgM antibodies. Lactoferrin-autoantibody positivity has previously been associated with disease activity in RA

(80), ankylosing spondylitis (81), systemic lupus erythematosus (82) and inflammatory bowel diseases (83), with a higher positivity for IgG autoantibodies against lactoferrin detected compared to IgM (82).

Nevertheless, one requirement for an effective autoantibody that plays a role in disease progression is the presence of the antigen on the cell membrane surface (84). To this end, we recorded an increase in lactoferrin on the outer surface of the ZZ-AATD neutrophil plasma membrane. In turn, the AATD cell was highly receptive to anti-lactoferrin antibody exposure, resulting in increased ROS production as measured by superoxide anion release. Simultaneous incubation of MM neutrophils with priming levels of TNF- α and purified anti-lactoferrin antibody was required to reach ROS levels similar to ZZ cells exposed to anti-lactoferrin antibody only.

To support *in vitro* results demonstrating the ability of AAT to modulate TNF- α signaling, we isolated plasma and neutrophils from ZZ-AATD patients who had been receiving AAT augmentation therapy for more than 4 years and a matched ZZ-AATD patient group who had never received AAT infusions. Results revealed that augmentation therapy significantly increased neutrophil membrane levels of AAT and decreased TNF- α surface membrane expression in neutrophils from patients receiving augmentation therapy, with the level of reduction (1.5 fold) observed similar to that recorded post-anti-TNF- α therapy in patients with RA (64). As plasma TNF- α has a short half-life (44-46), the evaluation of other surrogate plasma markers, such as soluble TNF R1 (47-49) was employed. Results confirmed reduced levels of TNF R1 only in patients receiving augmentation therapy when compared to patients not receiving treatment, thereby demonstrating that infused AAT modulates activation of the TNF- α system. In contrast however, a previous study reported no significant

difference between serum levels of TNF- α of patients receiving therapy compared to patients not receiving AAT augmentation treatment (85). Thus the results of the present study highlight the drawback of examining only serum or plasma levels of TNF- α . In addition, our results confirm the long-term efficacy of AAT augmentation therapy, as individuals receiving therapy for 4 years illustrated a significant decrease in titre of autoantibodies directed against lactoferrin.

The results of the present study analysing the effect of weekly infusions on TNF- α and neutrophil biology have identified a possible drawback of the current practice of weekly AAT infusions (60mg per kilo) to AATD patients. It has been established that weekly infusions of AAT augmentation therapy results in a peak and trough effect with regards to the AAT levels, resulting in initially normal to high levels of AAT in the circulation for several days after infusion. After this, however, the levels of AAT fall to near or below the protective threshold (3). The present study demonstrates that during the trough phase of augmentation therapy (day 7, AAT level of 7.59 μ M), neutrophils are in a primed state, as demonstrated by elevated TNF- α membrane levels and increased plasma concentrations of neutrophil degranulation markers. This part of the study specifically highlights the need for further studies evaluating dosing strategies in AAT augmentation therapy in order to maintain daily raised levels of AAT throughout treatment.

A number of limitations to this study should be discussed. Firstly, the absence of data evaluating the consequence of mid-level AAT plasma levels, as observed in MZ and SZ AATD phenotypes, on neutrophil function has not been addressed. This is an area of importance considering the high number of individuals whom are heterozygous for AATD (86, 87). Indeed, previous studies have indicated that

individuals with heterozygous AAT deficiency have an increased risk of developing lung disease when compared to healthy MM controls (88, 89). In addition, due to the recruitment criteria a second limitation of this study is the participation of a relatively small number of AATD patients receiving augmentation therapy. Despite this drawback however, the data provides evidence of the impact of infused AAT on TNF- α signaling in the circulating neutrophil.

In summary, our study has identified TNF- α as a potential parameter of disease progression and through the setting of AATD we have identified AAT as a natural modulator of TNF- α signaling. These results highlight the important role that AAT plays in regulating neutrophil biology and the possibility of AAT therapy as an additional TNF- α treatment strategy in management of patients with TNF- α driven diseases.

Materials and methods

Study Design

Ethical approval from Beaumont Hospital Institutional Review Board was acquired and written informed consent obtained from all study participants. Control volunteers (Table 1; N=10 subjects, mean age 28.3 ± 7.2 ; Table 2; N=30 subjects, mean age 35.2 ± 2.2) showed no evidence of any disease and had no respiratory symptoms; none were taking medication, all non-smokers and all proven MM phenotype with serum AAT concentrations within the normal range (25-50 μ M). ZZ-AATD patients were recruited from the Irish Alpha-1 Antitrypsin Deficiency Registry and were classified into three groups as follows:

1: Non-obstructed ZZ-AATD individuals, not receiving augmentation therapy (N=10 subjects, mean age 31.8 ± 16.5). Patients were clinically stable, non-smokers, with no evidence of exacerbations in the previous six months. Forced expiratory volume in one second (FEV₁) was $108.4 \pm 13.6\%$ predicted (Table 1).

2: Non-smoking ZZ-AATD patients who were not receiving augmentation therapy (N=30 subjects) with a FEV₁ of $74.7 \pm 6.3\%$ predicted (Table 2).

3: ZZ-AATD patients (non-smokers, N=4 subjects, mean age 56.5 ± 2.7 , FEV₁ $52.25\% \pm 8.27\%$ predicted) on augmentation therapy who were receiving plasma purified AAT from CSL Behring (Zemaira[®]), given intravenously at a dosage of 60mg/kg body weight weekly for 4 years. In the four weeks prior to obtaining blood samples, all patients were exacerbation free.

Chemicals and reagents

All chemicals and reagents used in this study were of the highest purity available, endotoxin free and were purchased from Sigma Aldrich unless otherwise indicated.

Plasma isolation

Blood was collected in Sarstedt Monovette tubes containing lithium-heparin. Plasma was immediately isolated by centrifugation of the blood (1000 x g, 10 min at room temperature) which was then aliquoted and stored at -80°C until required.

Purification and lysis of human neutrophils

Neutrophils were isolated from healthy control and ZZ-AATD whole blood by dextran sedimentation and Lymphoprep (Axis-Shield PoC) centrifugation as previously described (90). Purified cells were re-suspended in phosphate-buffered saline (PBS) (pH 7.4) containing 5mM glucose (PBSG) and used immediately. Purity of isolated neutrophils was validated by flow cytometry analysis using a monoclonal antibody against CD16b, a specific neutrophil marker (91). Neutrophil viability was assessed by trypan blue exclusion assay. Results confirmed viability of neutrophils above 98% and the purity of isolated neutrophils was greater than 96%.

Neutrophil plasma membranes were isolated by sucrose gradient subcellular fractionation as previously described (18). Whole cell lysates for Western blot analysis were prepared from neutrophils (10^7 /ml) using radio immunoprecipitation assay (RIPA) buffer (10mM Tris, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM $\text{Na}_4\text{P}_2\text{O}_7$, 2mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate) containing protease inhibitors (13 μ M aprotinin, 5mM benzamidine, 0.15mM N α -Tosyl-L-lysine chloromethyl ketone hydrochloride

(TLCK), 0.5mM N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 20mM N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSuc-AAPV-CMK), 10 μ M soybean trypsin inhibitor (SBTI), orthophenanthroline and 0.2M pefabloc) and phosphatase inhibitors (2mM sodium orthovanadate and 2mM sodium pyrophosphate).

Quantification of TNF- α , soluble TNF R1, lactoferrin, hCAP-18, MMP-9 and AAT in plasma

For the quantification of TNF- α plasma levels, the Human TNF-alpha Ultra-Sensitive Kit (Cat Number K151BHC-1) was employed according to the manufacturer's instructions (Meso Scale Discovery). Analysis of soluble TNF R1 (R&D Systems), lactoferrin and hCAP-18 was by sandwich ELISA (the latter two from Cambridge Bioscience). MMP-9 was quantified in plasma samples by zymography using novex gelatin zymogram gels (Invitrogen) according to the manufacturer's instructions. The MMP-9 (85kDa) band was visualised and quantified by densitometry using the Syngene G:BOX Chemi XL gel documentation system (92). AAT levels in plasma were determined by immune turbidimetry (Olympus AU5400).

Neutrophil degranulation assay

Purified neutrophils from healthy controls and/or non-obstructed ZZ-AATD patients were re-suspended in PBSG (2 $\times 10^7$ /ml). Cells were either unstimulated or stimulated with TNF- α (10ng) at 37°C in the presence or absence of increasing AAT concentrations (6.1, 13.25, 27.5, 55 or 110 μ M; Athens Research & Technology) for 0, 5, 10 or 20 min. Cell free supernatants were harvested following centrifugation at 500 x g for 5 min at 4°C and analysed for degranulated proteins by Western blotting. As a control the use of equal cell numbers (2 $\times 10^7$ /ml) in each reaction was demonstrated

by identical Coomassie blue stained electrophoretic profiles of whole cell lysates prepared from cells employed in each reaction (fig. S6 & S7).

SDS-PAGE and Western blot analysis

SDS-PAGE analyses of samples were carried out under denaturing conditions. Samples were electrophoresed using 12% NuPAGE® gels (Invitrogen) according to the manufacturer's instructions. After electrophoresis, gels were stained by Coomassie Blue R250 for visualization of proteins or alternatively proteins were transferred onto 0.2µm nitrocellulose or PVDF membrane by Western blotting using a semi dry blotter for 1 h at 100 mA. Efficient transfer was verified by Ponceau S staining of membrane followed by blocking the membrane in PBS-tween containing 3% (w/v) non-fat dried milk and 1% (w/v) bovine serum albumin (BSA). The following primary antibodies were utilized at a concentration of 1µg/ml; polyclonal goat anti-MMP-9 (R&D Systems); polyclonal rabbit anti-hCAP-18 (Invitrogen); polyclonal rabbit anti-lactoferrin; polyclonal rabbit anti-IκBα; polyclonal rabbit anti-GAPDH; monoclonal mouse anti-p38 MAPK (all from Santa Cruz Biotechnology), monoclonal anti-actin antibody (Millipore) and polyclonal rabbit anti-phospho-p38 MAPK (both from Cell Signaling Technology). Relative secondary antibodies were all horseradish peroxidase (HRP) linked anti-goat, rabbit or mouse (Cell Signaling Technology). Immunoreactive bands were visualized by utilising chemiluminescent HRP substrate (Millipore). Images and densitometry were obtained on the Syngene G:BOX Chemi XL gel documentation system.

Cell culturing

The HL-60 neutrophil like cell line (ATCC-CCL-240), were grown in complete RPMI media containing 10% (v/v) heat-inactivated fetal calf serum (FCS) and 1% (v/v)

penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂ until the required cell density was obtained. Cells (10⁷/ml) were washed in RPMI with no added FCS and resuspended in RPMI and either left untreated (control) or treated with TNF- α (2ng) in the presence or absence of physiological concentrations of AAT (27.5 μ M). NF κ B activation was assessed by Western blot analysis of I κ B α degradation in whole cell lysates of HL-60s (10⁷/ml) using RIPA Buffer. To quantify basal release of TNF- α by MM and ZZ-AATD cells, neutrophils were cultured for 6 h as previously described (18) with cell free supernatants analysed for TNF- α levels by ELISA (R&D Systems) employing the manufacturer's instructions.

Real time RT-PCR

TNF- α gene expression was assessed by real time RT-PCR as previously described (93), using SYBR Green I Master mix (Roche) with the LightCycler 480 PCR system (Roche). Primers for TNF- α (94) and GAPDH (95) were as previously described and obtained from MWG Biotech. PCR was performed using the following protocol: preincubation (95°C for 3 min); amplification [50 cycles consisting of denaturation, annealing, elongation (10 sec at 95°C; 10 sec at 57°C for both TNF- α and GAPDH; and 72°C for 10 sec); melting curve analysis (95°C for 5 sec, 65°C for 1 min, and 97°C for 5 continuous acquisitions)]; and final cooling step to 4°C. All PCRs were carried out in 96-well plates in 20 μ l reaction volumes and a negative control without cDNA was included in every run. The expression of target genes relative to GAPDH was determined using the 2^{- $\Delta\Delta$ C_T} method (96).

Flow cytometry analysis

Isolated neutrophils were fixed with 4% (w/v) paraformaldehyde for 10 min. Cells were then washed and blocked with 2% (w/v) BSA, followed by incubation with primary detection antibodies at a concentration of $1\mu\text{g}/10^6$ cells for 30 min. Primary antibodies consisted of mouse monoclonal anti-CD16b (Santa Cruz Biotechnology), monoclonal anti-TNF- α FITC (R&D Systems); mouse monoclonal anti-CD66b FITC (BD Biosciences), rabbit polyclonal anti-lactoferrin (Santa Cruz Biotechnology) and goat polyclonal anti-AAT FITC (Abcam). Control samples were exposed to relevant non-specific isotype control antibodies (goat IgG, rabbit IgG, mouse IgG₁ or IgM; all from Santa Cruz Biotechnology) or secondary FITC labeled antibodies alone (FITC goat anti-rabbit (Abcam) or FITC goat anti-mouse IgG (Santa Cruz Biotechnology)) (fig. S8).

In a subset of experiments, flow cytometry was employed to evaluate the ability of exogenous TNF- α to bind neutrophils as previously described (97). In brief, neutrophils were incubated with or without TNF- α (10ng) in the presence or absence of AAT (27.5 μM). After 10 min, cells were washed and then fixed and blocked as already described. TNF- α binding was quantified by incubating cells with a FITC labeled monoclonal antibody that can detect receptor bound TNF- α (R&D systems, $2\mu\text{g}/10^6$ cells) (fig. S9). Cells were washed in PBS and fluorescence counted using a BD FACSCalibur (Becton, Dickinson). Ten thousand events per reaction was quantified. Analysis of apoptosis and dead cells following TNF- α treatment was evaluated by using an Annexin V-FITC Apoptosis Kit (BioVision).

***In vitro* protein binding assays**

Analysis of the binding interaction of TNF- α with AAT was carried out using a surface-plasmon resonance assay for real-time and label-free detection. A positive

protein:protein interaction results in an increase in response units (RU). This was performed on a Biacore 2000 instrument using a CM-5 carboxymethylated dextran sensor chip (GE Healthcare). In brief; TNF- α (500ng/ml) was immobilized onto a CM-5 chip. Interaction between bound TNF- α and a mouse monoclonal anti-TNF- α antibody (R&D systems, 50nM; positive control) or AAT (5 μ M) was performed. All analyses were carried out in triplicate on 3 independent days, and binding responses (response unit [RU]) were monitored.

TNF- α and TNF receptor binding assays were carried out as previously described (18). Briefly, recombinant TNF receptor (TNFR1 or TNFR2; AbD Serotec) coated 96 well immunolon plates were exposed to TNF- α (10ng) in the presence or absence of AAT (27.5 μ M) or a TNF R1 Fc chimera (5nM) (TNF- α blocker, R&D Systems). A monoclonal anti-TNF- α antibody (R&D Systems) followed by HRP labeled rabbit anti mouse HRP antibody was utilised to detect a TNF- α binding event. Peroxidase activity was quantified by ABTS substrate and the resulting absorbance measured at 405nm.

A bead based assay was used to examine AAT and TNF receptor interaction as previously described (98). In brief, Polybeads (Polyscience Inc) were coated with either TNF R1 or TNF R2 (200nM) (R&D Systems) in Voller's buffer (15mM Na₂CO₃ and 35mM NaHCO₃, pH 9.3). As negative controls, beads were either unlabeled or coated with Fc γ RIIA (200nM) (R&D Systems) as AAT has previously been shown not to bind to this receptor (18). AAT (27.5 μ M) was incubated with the beads for 30 min at room temperature. Beads were washed and blocked with 2% (w/v) BSA in PBS. To confirm AAT interaction, flow cytometry was utilised by employing a FITC labeled polyclonal goat antibody against AAT (Abcam, 1 μ g/10⁶

beads). Relative isotype control antibody (FITC labelled goat IgG) was employed as a negative control.

Auto-antibody quantification and purification

An ELISA based method was utilised for the evaluation of autoantibodies against key secondary and tertiary granule proteins as previously described (53). In brief, purified lactoferrin, MMP-9 and hCAP-18 (10µg/ml) coated ELISA plates were incubated with plasma samples from MM healthy controls (N=30) and ZZ-AATD patients (N=30) (Table 2). The plates were washed and then incubated with a secondary rabbit anti-human IgG HRP or goat anti-human IgM HRP detection antibody (Sigma Aldrich) as per manufacturer's instructions. Peroxidase substrate ABTS was employed to determine positive signals within the assay. Three standard deviations of the healthy control group was used as a positive cut off point (53). Anti lactoferrin antibody from ZZ-AATD plasma was purified as previously described (99). The specificity of the purified antibody was confirmed by Western blot analysis. Purified human lactoferrin (1µg per lane) was electrophoresed and transferred to PVDF membrane. The purified anti-lactoferrin antibody was utilised as a primary antibody in the absence or presence of exogenous lactoferrin protein (5µg). Rabbit anti-human IgG HRP antibody was utilised as a detection antibody. Western blot signals were quantified by densitometry as described above.

Cytochrome C reduction assay

A cytochrome C reduction assay was employed to measure production of superoxide (O_2^-) by neutrophils as previously described (18). In brief, cells (5×10^5 /200µl) in cytochrome C buffer (100nM cytochrome C, 2mM $MgCl_2$, 2mM $CaCl_2$, 5mM glucose in PBS) were either untreated or treated with TNF- α as a priming agent (10ng)

followed by exposure to either rabbit anti-lactoferrin IgG antibody (1 μ g), the bacterial peptide fMLP (10⁻⁶M) or purified human anti-lactoferrin (300ng). The reduction of cytochrome was recorded at 550nm over 60 min.

Statistical analysis

Results are expressed as means \pm standard error of the mean (SEM) of biological replicates or independent experiments as stated in the figure legends. The data were analyzed with GraphPad Prism version 4.03 for Windows. Where appropriate a comparison of categorical data distributions by the D'Agostino–Pearson omnibus methods to test continuous data for normality of distribution was performed. The groups were compared by Student's t-test when normally distributed or by the nonparametric Mann–Whitney U test. Statistical comparisons of small data sets (N<6), a Student's t-test was employed (*100*). A value of $P < 0.05$ was considered statistically significant.

Supplementary Materials

Fig. S1. Equal tertiary and secondary granule protein expression in MM and ZZ-AATD neutrophils

Fig. S2. TNF- α exposure for 20 min does not induce apoptosis in MM or ZZ-AATD neutrophils

Fig. S3. Physiological and acute phase levels of AAT modulates TNF- α induced degranulation

Fig. S4. IgM autoantibodies against granule proteins in ZZ-AATD.

Fig. S5. Augmentation therapy does not impact upon soluble plasma levels of TNF R2.

Fig S6. Loading controls for Western blots analysing healthy control (MM) and ZZ-AATD neutrophil degranulation.

Fig. S7. Loading controls for Western blots analysing the effect of AAT compared to antithrombin III on neutrophil degranulation.

Fig. S8. Isotype controls for flow cytometry analysis of MM and ZZ-AATD neutrophils for TNF- α , lactoferrin, AAT and CD66b

Fig. S9. Isotype control for flow cytometry analysis of the ability of AAT to modulate neutrophil membrane TNF- α interaction.

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Table 1. Characteristics of healthy controls and non-obstructed ZZ-AATD patients employed in Figures 1-4.

	Non-Obstructed AATD	Control
No. of subjects	10	10
Gender, male/female	4/6	6/4
Age, years (Mean \pm SD)	31.8 \pm 16.5	28.3 \pm 7.2
FEV ₁ , % predicted	108.4 \pm 13.6	100.6 \pm 10.9
FVC, % predicted	114.9 \pm 19.9	105.7 \pm 16.6
FEV ₁ /FVC %	80.6 \pm 4.1	82 \pm 7.1
DLCO % predicted	85.7 \pm 11.7	-
BMI	24.85 \pm 7.0	26.9 \pm 4.8
Bronchiectasis on HRCT	2/10	-

Definition of abbreviations: SD = Standard deviation, FEV₁ = Forced expiratory volume in one second, FVC = Forced vital capacity, DLCO = Diffusion capacity for carbon monoxide, BMI = body mass index, HRCT = High resolution computer tomography.

Table 2. Characteristics of healthy control (MM) and ZZ-AATD patients used in autoantibody analysis in Figure 4a-c.

	MM Healthy controls	ZZ-AATD
No. of subjects	30	30
Gender, male/female	14/16	16/14
Age, years (Mean \pm SD)	35.2 \pm 2.2	47.5 \pm 2.9
FEV ₁ , % predicted	99.8 \pm 5.8	74.7 \pm 6.3

Definition of abbreviations: SD = Standard deviation, FEV₁ = Forced expiratory volume in one second.

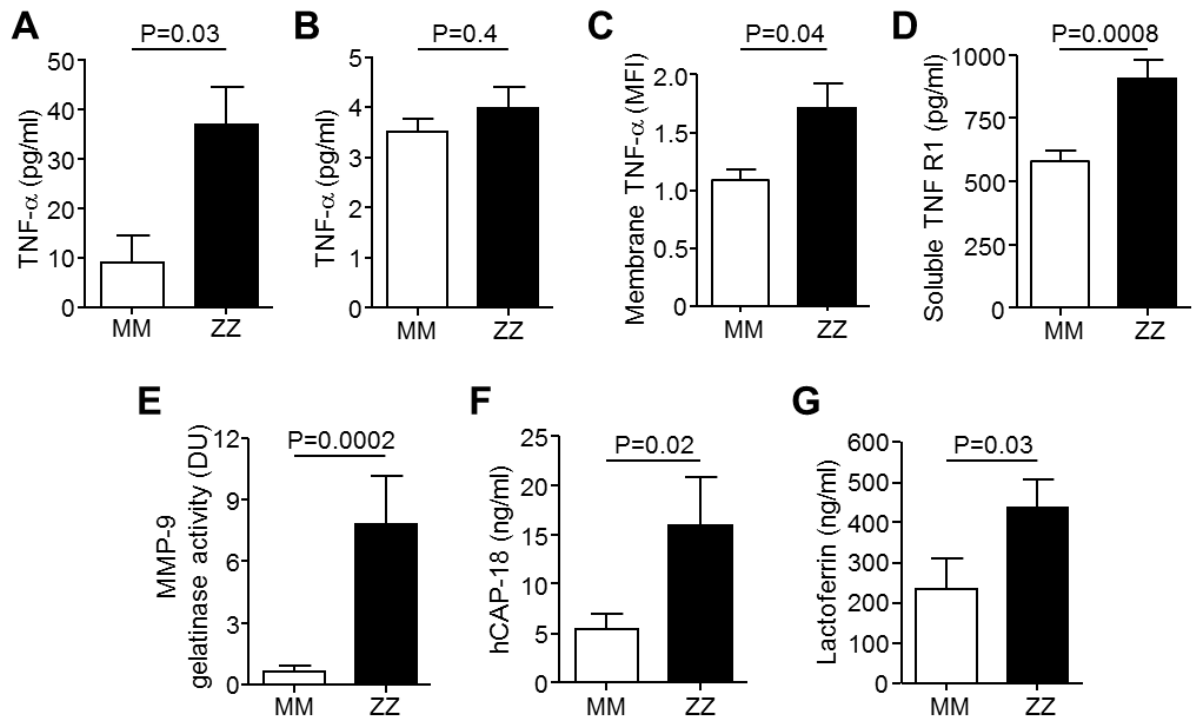


Fig. 1. TNF-α and neutrophil secondary and tertiary granule proteins in ZZ-AATD.

(A) TNF-α in cell supernatants from cultured peripheral blood neutrophils isolated from healthy control donors (MM) and ZZ-AATD patients (ZZ) was quantified by ELISA. Levels were significantly elevated in ZZ-AATD compared to the MM control group ($P=0.03$, $N=5$ subjects per group, Student's t-test). (B) No significant difference was observed in plasma levels of TNF-α between ZZ-AATD patients and MM healthy controls ($P=0.4$, $N=10$ subjects per group, Student's t-test). (C) Analysis of TNF-α revealed a significant increase in membrane expression on ZZ-AATD neutrophils when compared to control MM cells ($P=0.04$, $N=3$ subjects per group, Student's t-test). (D) Levels of soluble TNF R1 were significantly higher in ZZ-AATD plasma samples when compared to MM controls ($P=0.0008$, $N=10$ subjects per group, Student's t-test). (E) Levels of MMP-9 were quantified by zymography and expressed as relative densitometry units (DU; $P=0.0002$, $N=10$ subjects per group, Student's t-test), (F) hCAP-18 ($P=0.02$, $N=10$ subjects per group, Mann-Whitney U test) and (G) lactoferrin ($P=0.03$, $N=10$ subjects per group, Student's t-test) quantified by ELISA and expressed as ng/ml, were significantly higher in plasma of ZZ-AATD individuals compared to MM healthy donors. All measurements are mean \pm SEM from biological replicates.

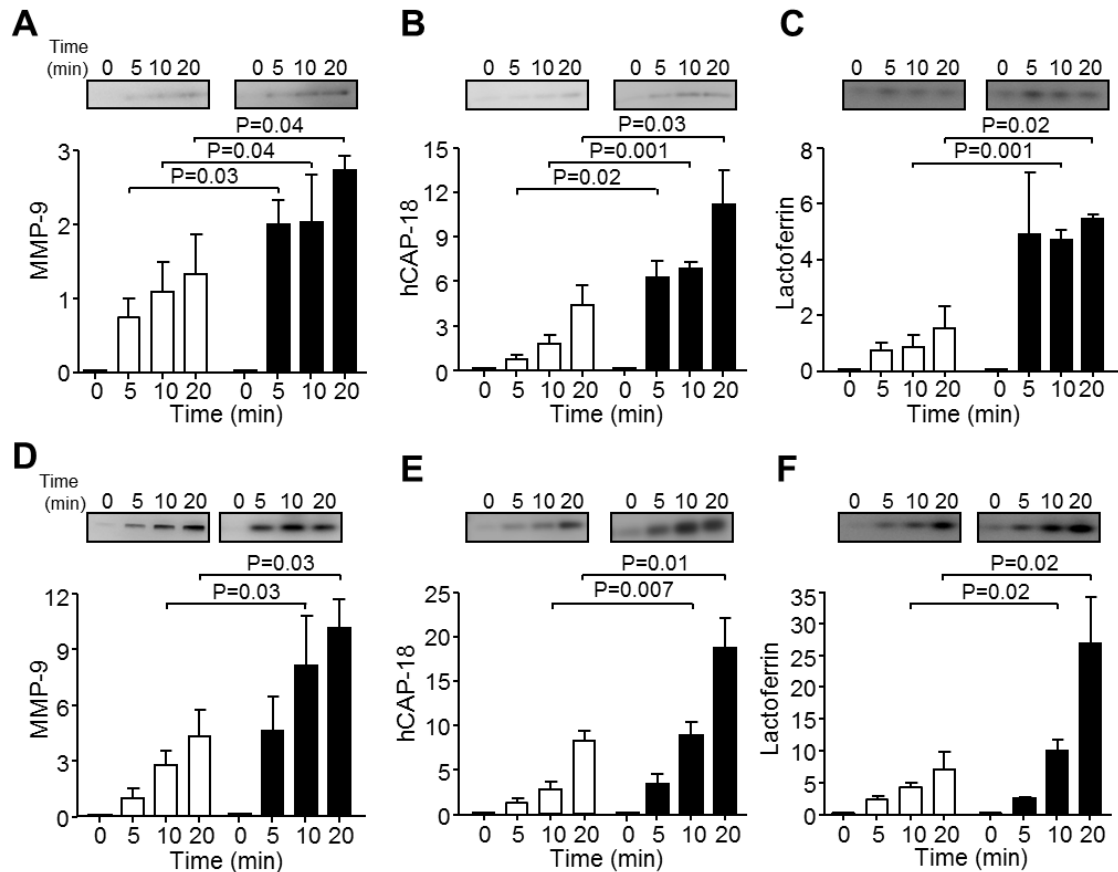


Fig. 2. Increased degranulation of secondary and tertiary granules by ZZ-AATD neutrophils in response to TNF- α .

Neutrophils isolated from MM (□) or ZZ-AATD (■) individuals were incubated at 37°C and were either unstimulated (A-C) or treated with TNF- α (D-F). Cell free supernatants were collected at 0, 5, 10 or 20 min and Western blotted for markers of tertiary granule [MMP-9 (A&D)] or secondary granule [hCAP-18 (B&E) and lactoferrin (C&F)] release. ZZ-AATD neutrophils released significantly greater levels of both granule types when compared to MM cells ($P < 0.05$, $N = 3$ subjects per group, Student's t-test). All results (expressed as relative densitometry units) are representative of three independent experiments. All measurements are mean \pm SEM from biological replicates.

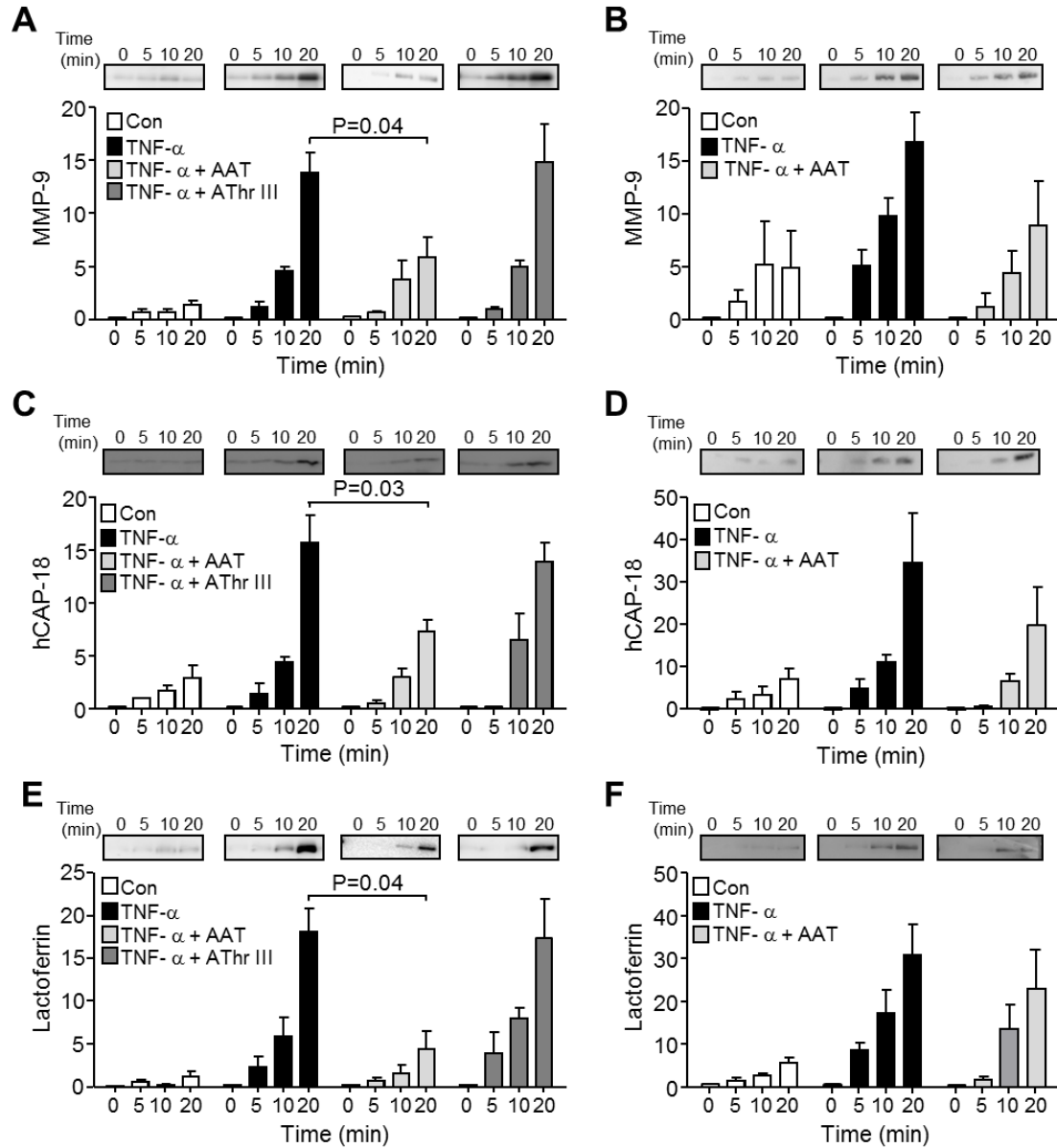


Fig. 3. AAT modulates TNF- α induced neutrophil degranulation.

Neutrophils were isolated from MM healthy controls (**A**, **C** & **E**) and asymptomatic ZZ-AATD patients (**B**, **D** & **F**). Cells were either unstimulated (Con) or stimulated with TNF- α in the absence or presence of either AAT (27.5 μ M) or antithrombin III (AThr III, 4 μ M). Supernatants were analysed by Western blotting to determine neutrophil degranulation. AAT significantly reduced degranulation of MMP-9 (**A**), hCAP-18 (**C**) and lactoferrin (**E**) by MM neutrophils stimulated with TNF- α after 20 min, while AThr III had no effect ($P < 0.05$ between TNF- α and TNF- α with AAT at the respective time points, $N = 3$ subjects per group, Student's t-test). Physiological levels of AAT (27.5 μ M) had no significant impact on TNF- α induce degranulation of MMP-9 (**B**), hCAP-18 (**D**) and lactoferrin (**F**) from asymptomatic ZZ-AATD neutrophils. All results are expressed as relative densitometry units. All measurements are mean \pm SEM from biological replicates.

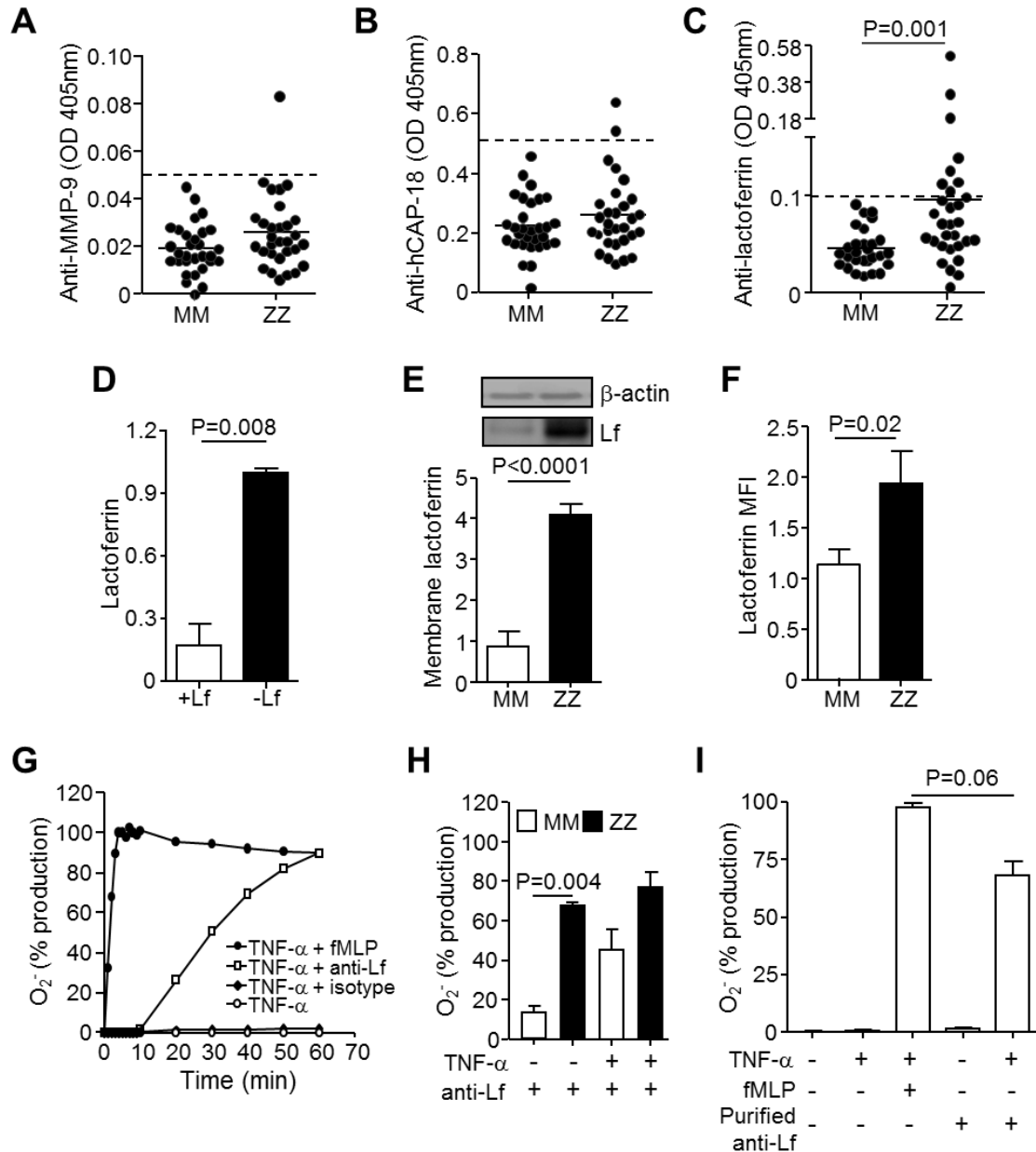


Fig. 4. Autoantibodies in ZZ-AATD are associated with increased neutrophil activation and ROS production.

IgG autoantibodies against MMP-9 (A), hCAP-18 (B) and lactoferrin (C) were quantified in plasma of MM healthy controls and ZZ-AATD individuals. A significant increase in the titre of lactoferrin autoantibodies were detected in ZZ-AATD patients ($P=0.001$, $N=30$ subjects per group, Mann-Whitney U test). Positivity was set as 3 standard deviations above the mean of MM healthy controls as indicated by the hatched line. 1, 2 and 8 of 30 ZZ-AATD samples were positive for MMP-9, hCAP-18 and lactoferrin autoantibodies, respectively. (D) The specificity of purified anti-lactoferrin antibody from patient's sera was evaluated by Western blot analysis. The presence of exogenous lactoferrin (Lf) significantly reduced the ability of the purified human anti-lactoferrin to detect PVDF membrane immobilised lactoferrin ($P<0.0001$, $N=3$ independent experiments, Student's t-test). By Western blot (employing β -actin as a loading control) (E) and flow cytometry analysis (F), significantly increased levels of degranulated lactoferrin (Lf) were found bound to the outer surface of ZZ-

AATD neutrophil plasma membranes compared to MM control cells ($P < 0.0001$ and $P = 0.02$ respectively, $N = 3$ subjects per group, Student's t-test). **(G)** TNF- α primed MM control neutrophils (5×10^5) were fMLP (10^{-6} M) stimulated and the rate of O_2^- generation compared to stimulation by lactoferrin autoantibodies (anti-Lf, $1 \mu\text{g}$). Controls for this experiment included cells exposed to TNF- α only (10ng) or cells exposed to isotype control antibody ($1 \mu\text{g}$). **(H)** A comparison of O_2^- generated by MM and ZZ-AATD cells post 30 min exposure to lactoferrin autoantibodies (anti-Lf, $1 \mu\text{g}$) in the presence or absence of TNF- α (10ng). Lactoferrin antibodies induced significantly more O_2^- release from ZZ-AATD neutrophils compared to MM cells ($P = 0.004$, $N = 3$ subjects per group, Student's t-test). **(I)** TNF- α (10ng for 10min) primed MM control neutrophils (5×10^5) were stimulated by fMLP (10^{-6} M) or purified plasma ZZ-AATD lactoferrin autoantibodies (Purified anti-Lf, 300ng) and the rate of O_2^- generation compared at 60 min was not statistically significant ($P = 0.06$, $N = 3$ MM subjects, Student's t-test). Controls included untreated cells or those exposed to either TNF- α or purified autoantibody only (300ng). O_2^- is expressed as a % of the response to TNF- α and fMLP. Each measurement in **E**, **F**, **H** and **I** are the mean \pm SEM from biological replicates.

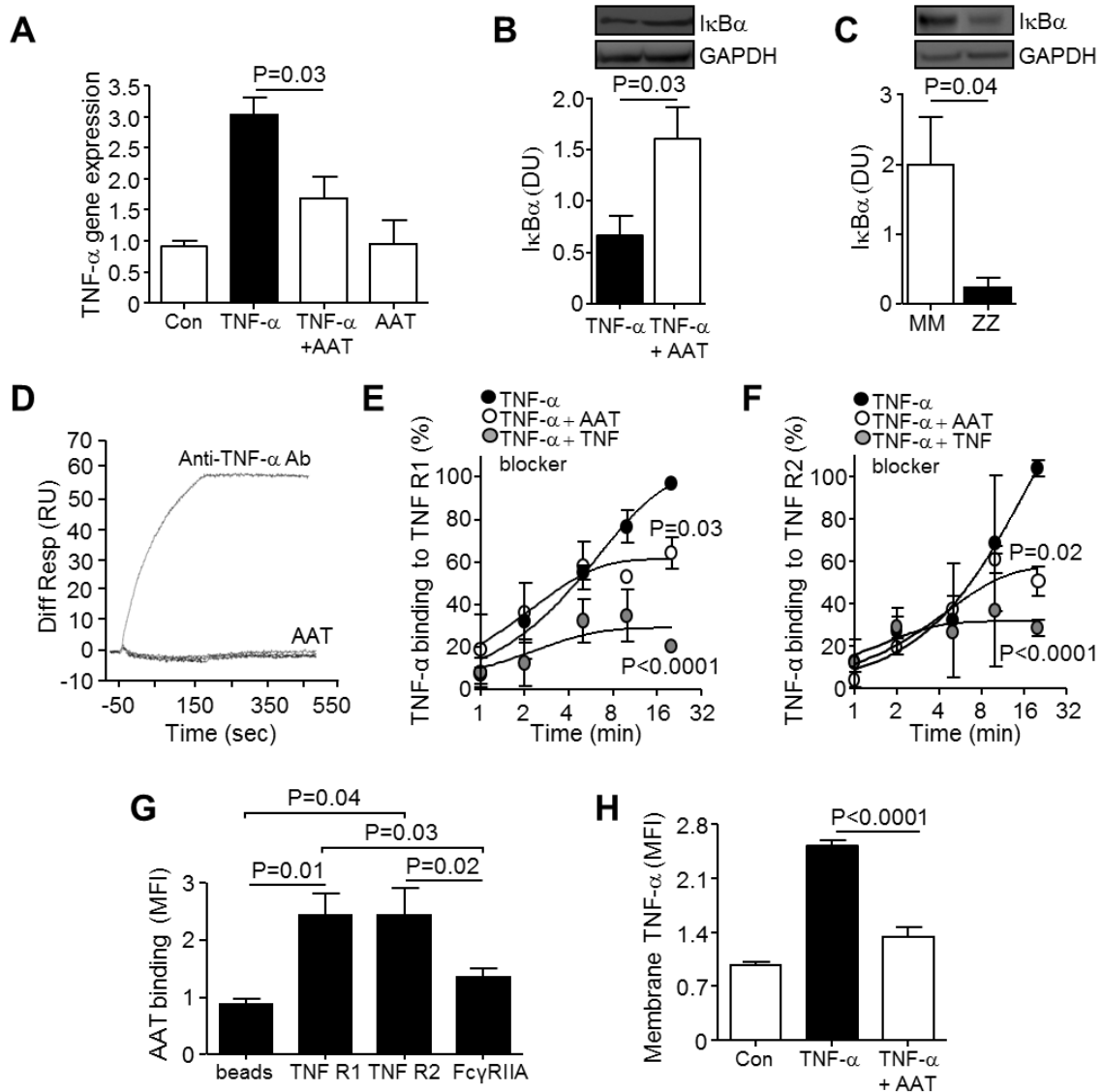


Fig. 5. AAT modulates TNF- α signaling by affecting TNF- α , TNF R1 and TNF R2 interaction.

(A) TNF- α qRT-PCR results of HL-60 cell RNA post-exposure to TNF- α or TNF- α and AAT (27.5 μ M) for 6 h. TNF- α gene expression was significantly inhibited when incubated in the presence of AAT (P=0.03, N=3 independent experiments, Student's t-test). (B) Western blot densitometry analysis of HL-60 cells treated for 20 min with TNF- α \pm AAT (27.5 μ M) normalized to GAPDH. Results in arbitrary densitometry units (DU) indicate that TNF- α exposure caused decreased levels of I κ B α , indicative of NF- κ B activation. I κ B α degradation was reduced by the addition of AAT (P=0.03, N=3 independent experiments, Student's t-test). (C) Western blot densitometry analysis of MM and ZZ-AATD neutrophil whole cell lysates. Significantly reduced I κ B α levels were detected in the ZZ-AATD samples as compared to healthy MM control cells (P=0.04, N=5 subjects per group, Student's t-test). (D) Biacore analysis indicates that AAT does not interact directly with TNF- α . An anti-TNF- α monoclonal antibody was used as a positive control to demonstrate a binding event. Analysis of TNF- α interaction with immobilised TNF R1 (E) and TNF R2 (F) in the absence or presence of AAT or a TNF- α blocker. Results indicate that AAT reduced TNF- α

binding to TNF R1 and R2 by 40 % ($P=0.03$, $N=3$ independent experiments, Student's t-test) and 50 % ($P=0.02$, $N=3$ independent experiments, Student's t-test), respectively. Similarly the TNF- α blocker caused a significant reduction in TNF- α binding to TNF R1 and R2 ($P<0.0001$ and $P<0.0001$, $N=3$ independent experiments, Student's t-test). (**G**) Flow cytometry analysis demonstrates that AAT interacts with TNF R1 and TNF R2 coated polybeads when compared to beads alone ($P=0.01$ and $P=0.04$, $N=3$ independent experiments, Student's t-test) or Fc γ RIIA coated beads ($P=0.03$ and $P=0.02$, $N=3$ independent experiments, Student's t-test). (**H**) Analysis of the ability of exogenous TNF- α to bind to the neutrophil was examined by flow cytometry. Cells incubated with AAT (27.5 μ M) and exposed to TNF- α displayed a significant reduction in the mean fluorescence intensity units (MFI) when compared to cells incubated with TNF- α only ($P<0.0001$, $N=3$ MM subjects, Student's t-test). Each measurement in **A-C** and **H** is the mean \pm SEM from biological replicates. Image **D** is a representative result from three independent experiments.

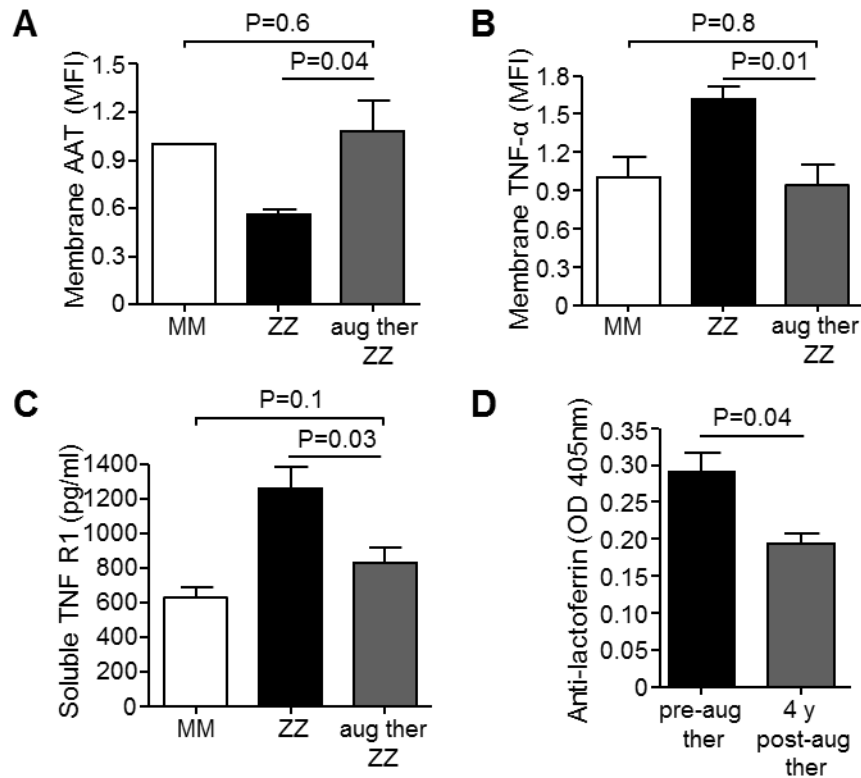


Fig. 6. Augmentation therapy impacts on neutrophil membrane proteins and autoimmunity.

Neutrophils and plasma were isolated from healthy controls (MM), ZZ-AATD patients receiving augmentation therapy for over 4 years (aug ther ZZ) or ZZ-AATD patients not receiving augmentation therapy (ZZ). Flow cytometry analysis for membrane bound AAT (A) or TNF-α (B) demonstrated that augmentation therapy significantly increased membrane AAT yet decreased membrane TNF-α levels in ZZ-AATD patients when compared to patients not receiving therapy (P=0.04 and P=0.01, N=3 subjects per group, Student's t-test). Membrane levels of AAT and TNF-α were not significantly different between the MM group and ZZ-AATD patients receiving therapy (P=0.6 and P=0.8 respectively, N=3 subjects per group, Student's t-test). Results expressed as mean fluorescence intensity (MFI). (C) ZZ-AATD patients receiving augmentation therapy demonstrated a significant decrease in soluble plasma TNF R1 compared to ZZ-AATD patients not receiving augmentation therapy (P=0.03, N=3 subjects per group, Student's t-test), but similar to levels in MM control plasma samples (P=0.1, N=3 subjects per group, Student's t-test). (D) IgG autoantibodies against lactoferrin were quantified in plasma of ZZ-AATD individuals prior to receiving augmentation therapy (pre-aug ther) and from the same individuals 4 years post treatment (4 y post-aug ther). A significant decrease in the titre of lactoferrin autoantibodies was detected in ZZ-AATD patients (P=0.04, N=4 subjects per group, Student's t-test). Each measurement is the mean ± SEM from biological replicates.

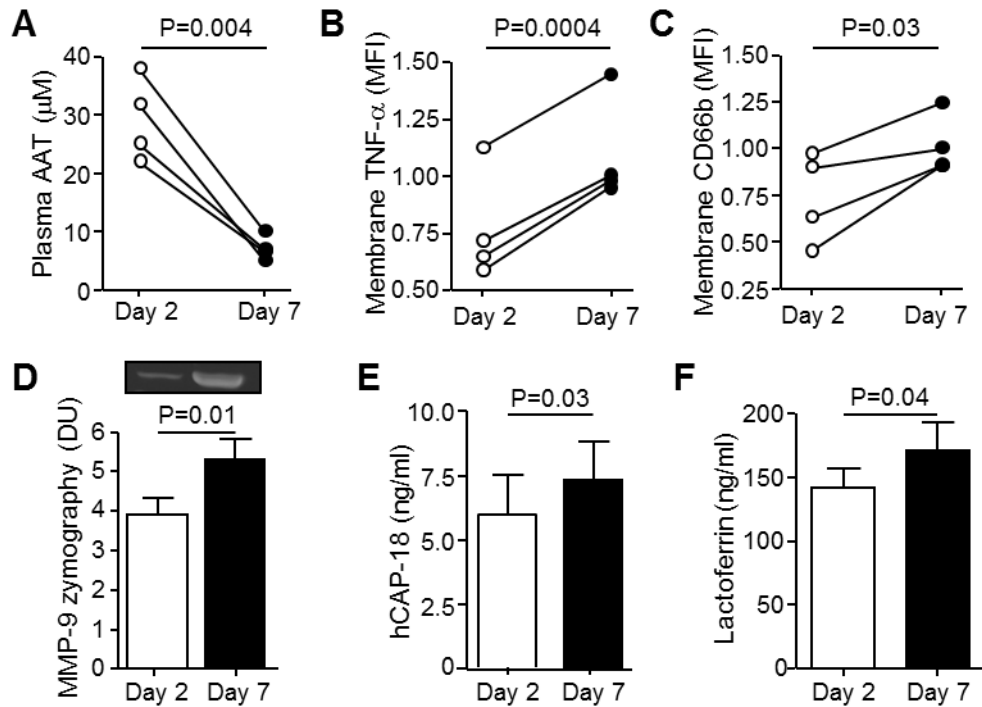


Fig. 7. Impact of weekly augmentation therapy infusions on AAT, TNF- α and plasma granule protein levels.

(A) Increased plasma levels of AAT on day 2 (\circ) compared to day 7 post (\bullet) augmentation therapy in ZZ-AATD patients ($P=0.004$, $N=4$ patients, Student's t-test). Flow cytometry analysis of membrane-bound TNF- α (B), or CD66b (C), on isolated ZZ-AATD neutrophils on day 2 and 7 post-augmentation therapy. Results in mean fluorescence intensity units (MFI) demonstrate significantly reduced levels 2 days post-augmentation therapy compared to day 7 ($P=0.0004$ and $P=0.03$, $N=4$ paired patient samples, Student's t-test). (D-F) Levels of active MMP-9 (D; expressed as arbitrary densitometry units (DU)), hCAP-18 (E) and lactoferrin (F) were significantly lower in plasma of ZZ-AATD individuals on day 2 post infusion compared to day 7 post therapy ($P=0.01$, $P=0.03$ and $P=0.04$ respectively, $N=4$ patients, Student's t-test). Zymography gel in D is 1 of 3 separate experiments. Each measurement in D-F is the mean \pm SEM from biological replicates.

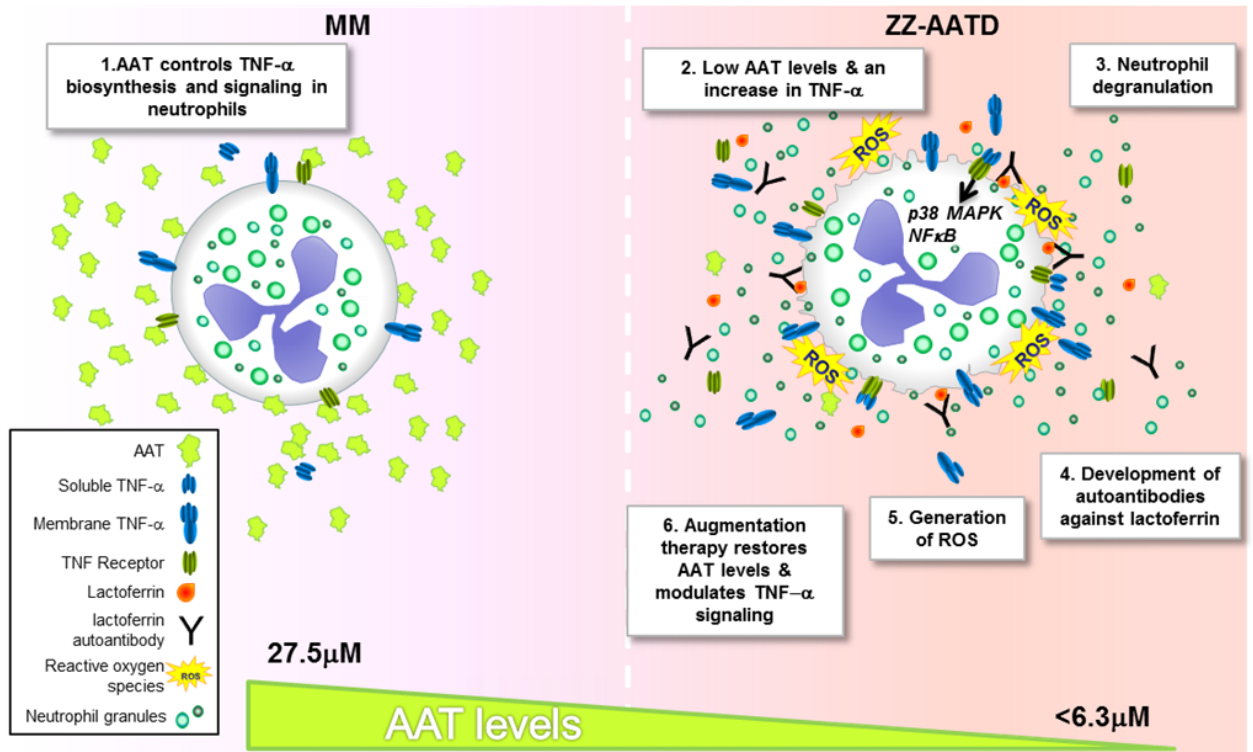
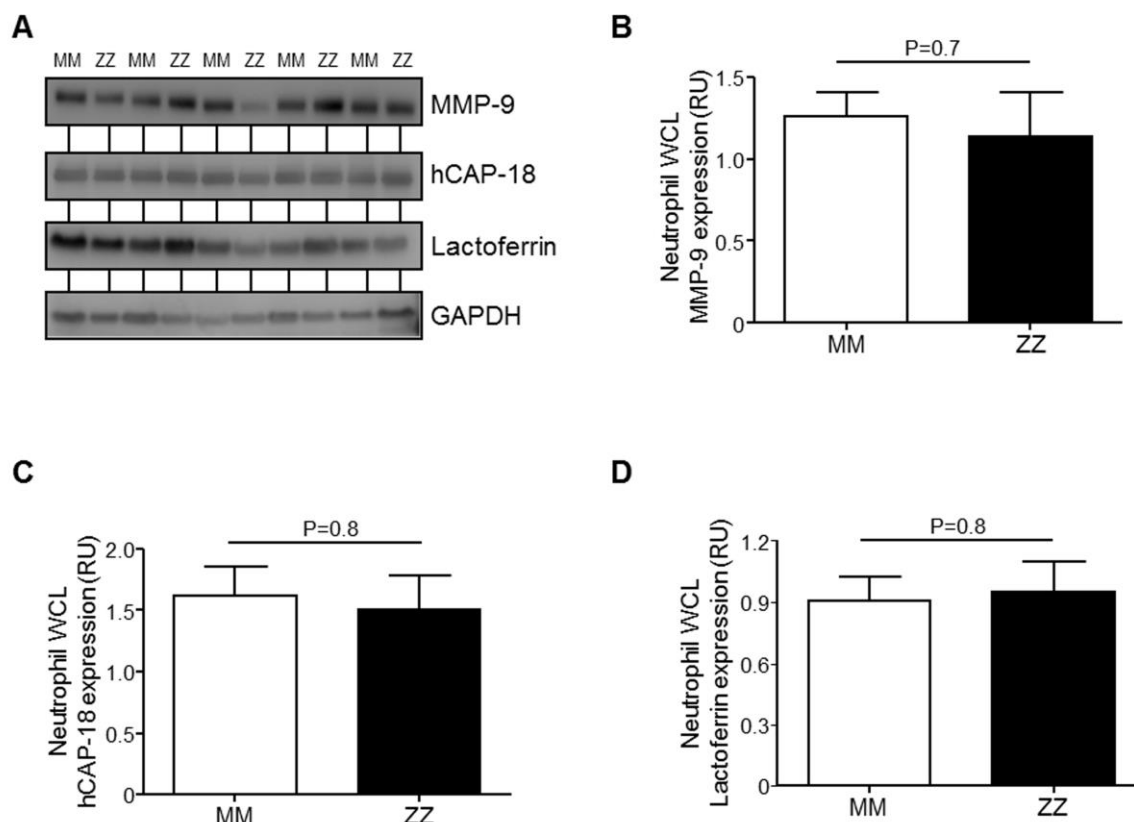


Fig. 8. AAT modulates TNF- α signaling in neutrophils.

Physiological serum levels of AAT modulate TNF- α signaling (1). In ZZ-AATD, the low level of AAT results in increased TNF- α signaling (2), neutrophil priming and increased degranulation of secondary and tertiary granules (3). The described excessive degranulation can lead to development of autoantibodies against released granule proteins including lactoferrin (4). These autoantibodies can lead to activation of the neutrophil in ZZ-AATD resulting in production of ROS (5). Augmentation therapy restores the concentration of AAT in the circulation to normal levels, thereby modulating TNF- α signaling, preventing neutrophil degranulation and averting an autoimmune response (6).

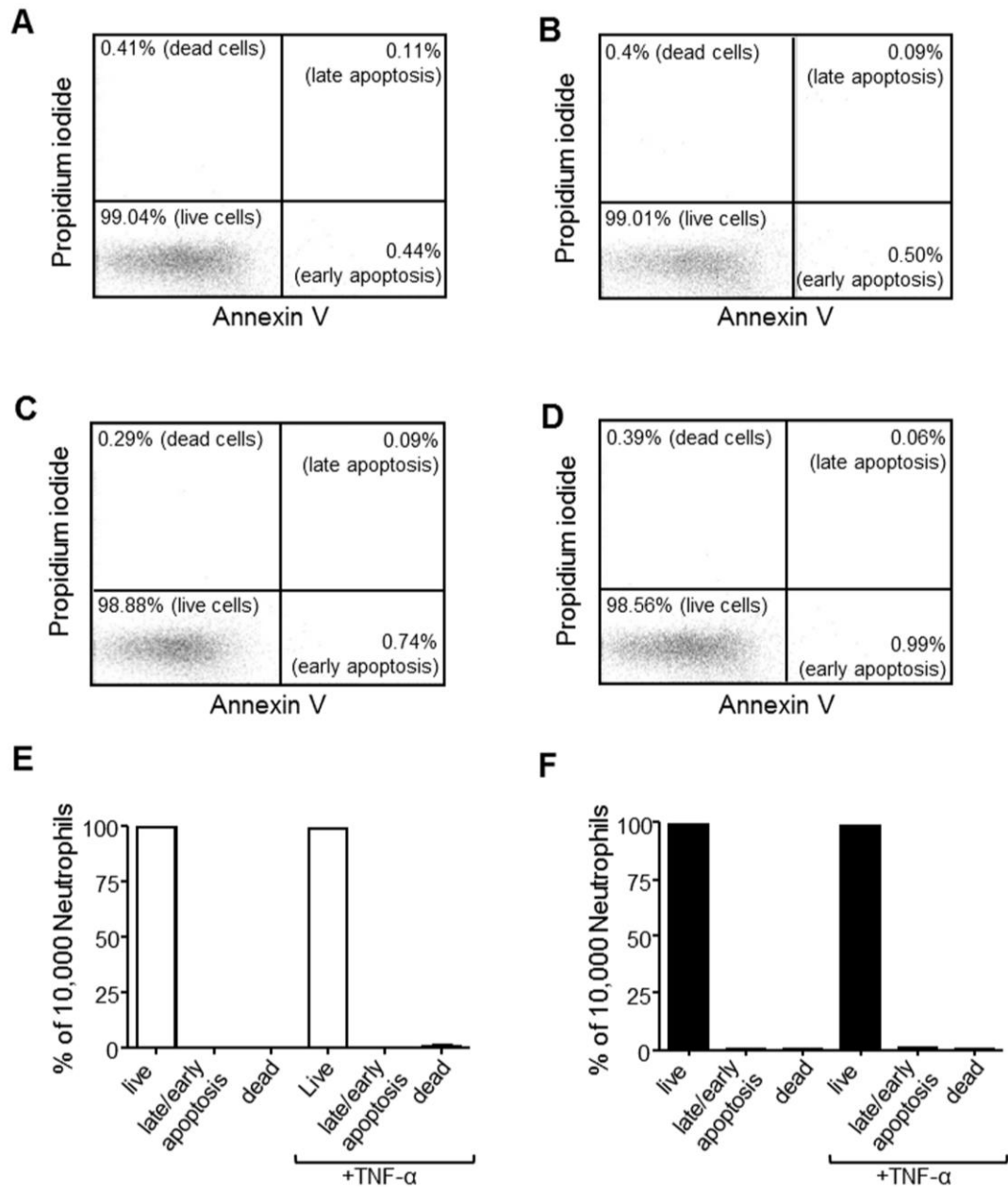
Figure S1



Equal tertiary and secondary granule protein expression in MM and ZZ-AATD neutrophils

(A) Western blots of whole cell lysates (WCL) prepared from healthy control (MM) and ZZ-AATD (ZZ) neutrophils probed for MMP-9, hCAP-18, lactoferrin and GAPDH (loading control). (B) Densitometry values of the expression levels of MMP-9 ($P=0.7$, $N=5$ subjects per group, Student's t-test), (C) hCAP-18 ($P=0.8$, $N=5$ subjects per group, Student's t-test) and (D) lactoferrin ($P=0.8$, $N=5$ subjects per group, Student's t-test) were not statistically significant between MM and ZZ-AATD. Each measurement is the mean \pm SEM from biological replicates.

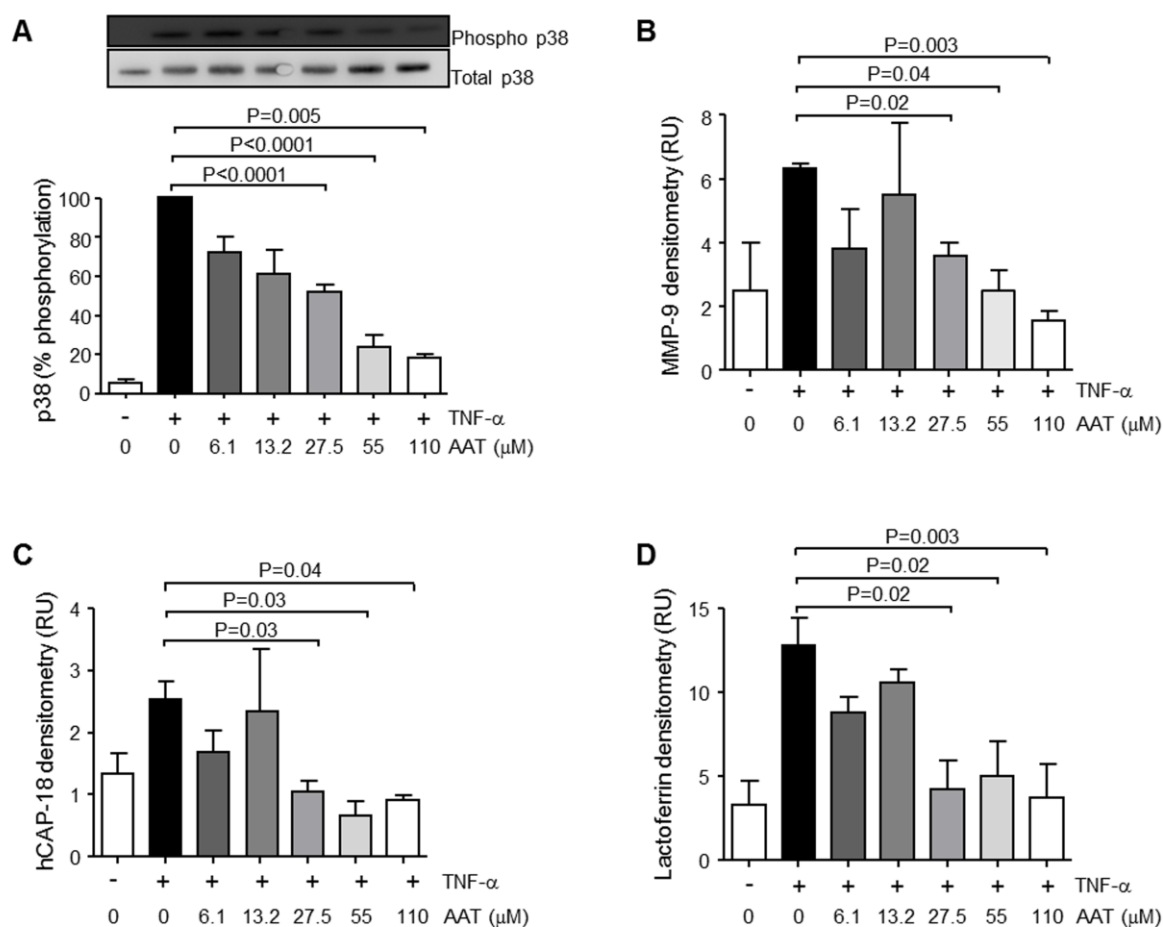
**Figure
S2**



TNF-α exposure for 20 min does not induce apoptosis in MM or ZZ-AATD neutrophils

(A-D) MM and ZZ-AATD neutrophils incubated with or without TNF-α (10ng/2x10⁷ cells) for 20 min at 37°C. Annexin V staining was performed to quantify apoptosis and propidium iodide (PI) staining to indicate the number of dead cells (% of 10,000 cells). Panels (A-D) show representative dot plots of dual annexin V (X-axis) and PI (y-axis) staining. (A) demonstrates dual staining of MM unstimulated neutrophils and (B) MM cells cultured with TNF-α for 20 min, (C) ZZ-AATD neutrophils unstimulated and (D) cultured with TNF-α for 20 min. (E&F) Bar graphs demonstrating the percentage of live, apoptotic (early and late) and dead cells (PI) for MM ± TNF-α (E) and ZZ-AATD ± TNF-α (F). Each measurement is the mean ± SEM from 3 biological replicates.

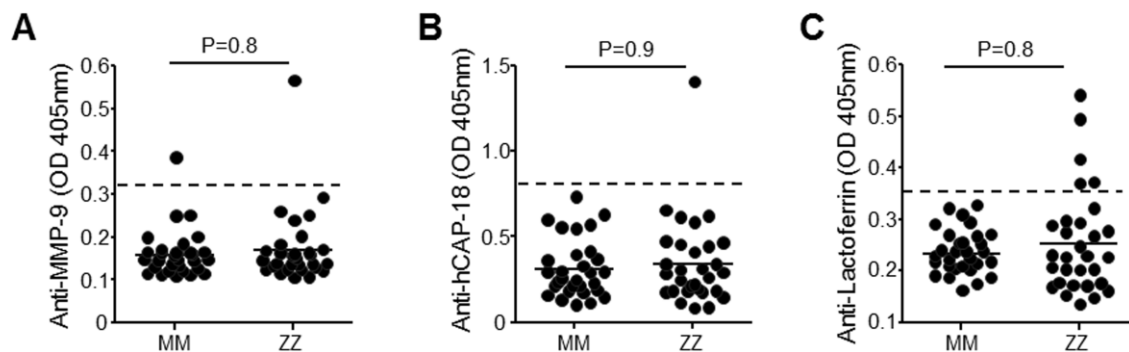
Figure S3



Physiological and acute phase levels of AAT modulates TNF- α induced degranulation

(A) Expression levels of phospho- MAP kinase p38 post TNF- α (2.5ng/1x10⁷ cells) treatment in neutrophil lysates were measured by Western blot analysis. AAT significantly inhibited MAP kinase p38 activity with an IC₅₀ of 29.15 μ M. AAT levels (27.5-110 μ M) significantly reduced p38 phosphorylation (P<0.0001, P<0.0001 and P=0.005 for 27.5 μ M, 55 μ M and 110 μ M AAT respectively, N=3 MM subjects, Student's t-test). Western blot analysis and densitometry of degranulated markers MMP-9 (B), hCAP-18 (C) and lactoferrin (D) in response to TNF- α in the presence of increasing concentrations of AAT. Results revealed that physiological levels of AAT (27.5 μ M) and up to acute phase levels of AAT (110 μ M) significantly reduced neutrophil degranulation (N=3 MM subjects, Student's t-test). Each measurement is the mean \pm SEM from biological replicates.

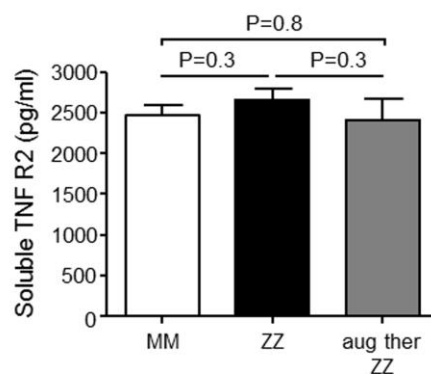
Figure S4



IgM autoantibodies against granule proteins in ZZ-AATD.

IgM autoantibodies against MMP-9 (A), hCAP-18 (B) and lactoferrin (C) were quantified in plasma of MM healthy controls and ZZ-AATD individuals. No significant difference in IgM titres were observed for MMP-9, hCAP-18 or lactoferrin between MM and ZZ ($P=0.8$, $P=0.9$ and $P=0.8$, respectively, $N=30$ subjects per group, Mann-Whitney U test). Positivity was set as 3 standard deviations above the mean of MM healthy controls as indicated by the hatched line. Of the ZZ-AATD samples 1/30, 1/30 and 5/30 were positive for MMP-9, hCAP-18 and lactoferrin autoantibodies, respectively.

Figure S5



Augmentation therapy does not impact upon soluble plasma levels of TNF R2.

Soluble TNF R2 was quantified in plasma from healthy control (MM), ZZ AATD (FEV₁ 30-80%, ZZ) and ZZ-AATD patients receiving augmentation therapy (aug ther ZZ) (FEV₁ 30-80%) by ELISA. Results demonstrated no significant difference in soluble plasma levels of TNF R2 between the three groups (MM versus ZZ or ZZ versus aug ther ZZ, P=0.3; MM versus aug ther ZZ, P=0.8. N=3 subjects per group, Student's t-test). Each measurement is the mean \pm SEM from biological replicates.

A

MM ZZ-AATD

0 5 10 20 0 5 10 20 Time (min)

Coomassie gel

Blots

MMP-9

hCAP-18

Lactoferrin

B

MM ZZ-AATD

0 5 10 20 0 5 10 20 Time (min)

Coomassie gel

Blots

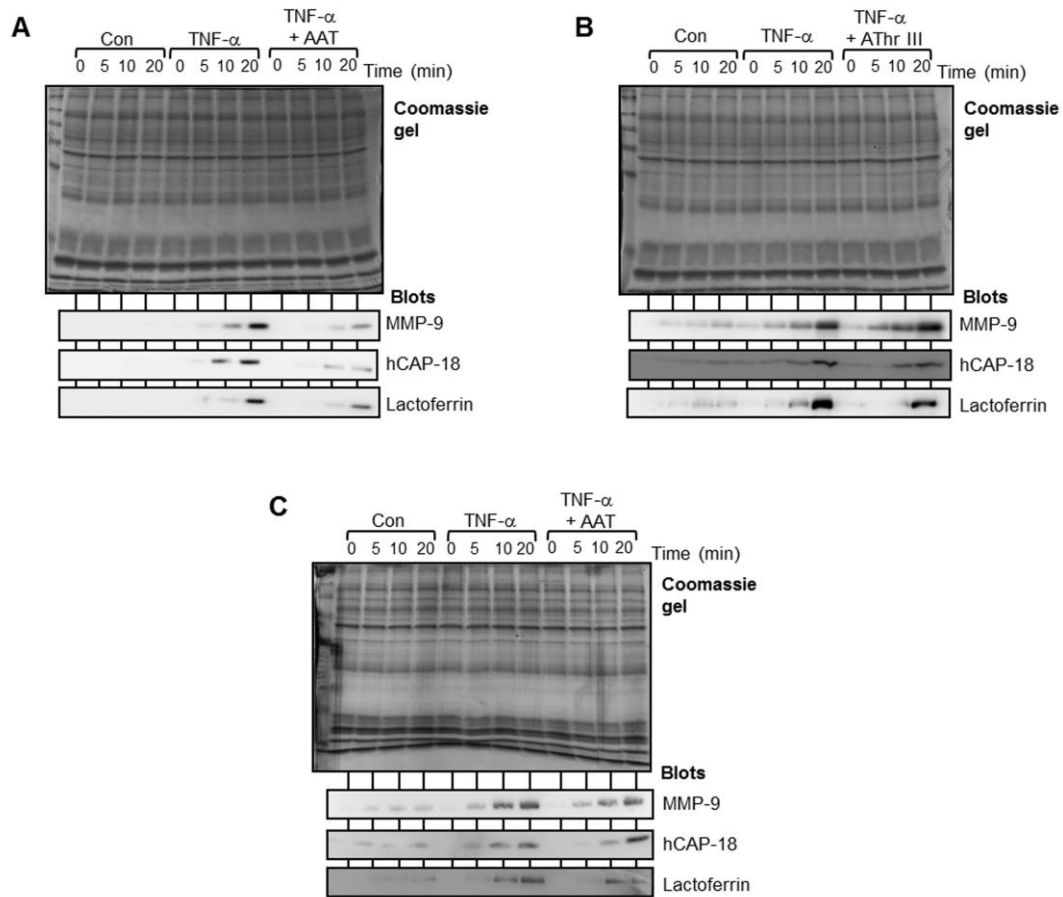
MMP-9

hCAP-18

Lactoferrin

An evaluation of tertiary (MMP-9) and secondary granule (hCAP-18 & Lactoferrin) release from unstimulated (**A**) and TNF- α stimulated (**B**) MM and ZZ-AATD neutrophils. The use of equal cell numbers in each reaction is demonstrated by the identical electrophoretic profile of whole cell lysates in the Coomassie blue stained gels (loading control). The Coomassie gels and corresponding Western blots are representative of 3 experiments.

Figure S7



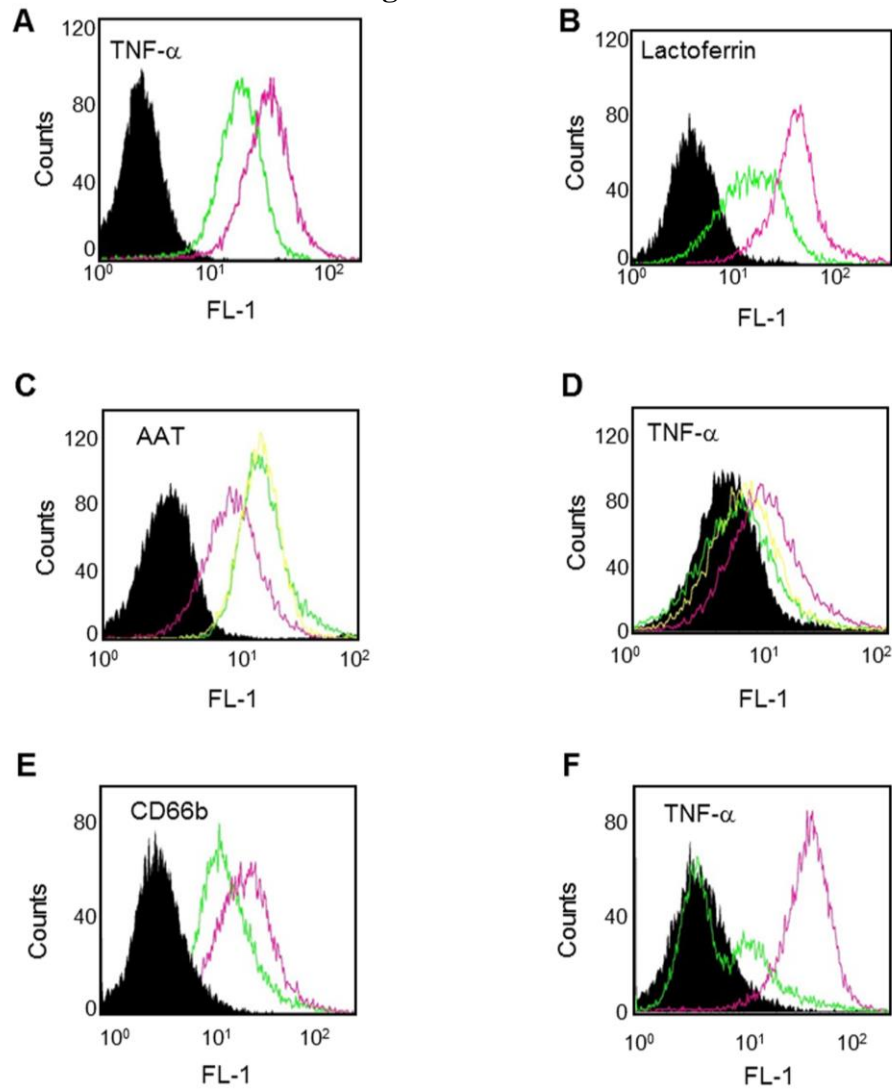
Loading controls for Western blots analysing the effect of AAT compared to antithrombin III on neutrophil degranulation.

(A) An evaluation of tertiary (MMP-9) and secondary granule (hCAP-18 & lactoferrin) release by MM cells treated with TNF- α \pm AAT or (B) TNF- α \pm antithrombin III (AThrIII). The use of equal cell numbers in each reaction is demonstrated by identical electrophoretic profiles of whole cell lysates in the Coomassie blue stained gel (loading control).

(C) Impact of AAT on TNF- α induced neutrophil degranulation in ZZ-AATD cells. The use of equal cell numbers in each reaction is demonstrated by the identical electrophoretic profile of whole cell lysates in the Coomassie blue stained gel (loading control).

The Coomassie gels and Western blots are representative of 3 experiments.

Figure S8



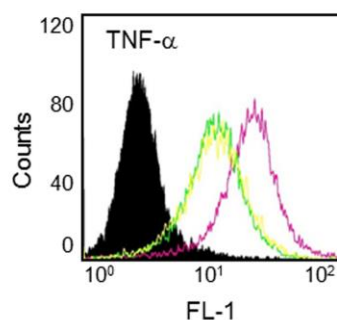
Isotype controls for flow cytometry analysis of MM and ZZ-AATD neutrophils for TNF- α , lactoferrin, AAT and CD66b

(A) Expression levels of TNF- α and (B), lactoferrin on MM control (green histogram) and non-obstructed ZZ-AATD neutrophils (purple histogram). Relative isotype control antibodies, mouse IgG₁ (A) and rabbit IgG (B) are illustrated (black filled histogram).

(C) Membrane expression levels of AAT and (D), TNF- α , on neutrophils of MM controls (yellow histogram), ZZ-AATD patients day 2 post augmentation therapy (green histogram) and ZZ-AATD patients who never received augmentation therapy (purple histogram). Relative isotype control antibodies, goat IgG (C) and mouse IgG₁ (D) are illustrated (black filled histogram).

(E) The impact of augmentation therapy on levels of membrane-bound CD66b and (F), TNF- α on isolated ZZ-AATD neutrophils on day 2 (green histogram) and 7 days post-augmentation therapy (purple histogram). Relative isotype control antibodies, mouse IgM (E) and mouse IgG₁ (F) are represented in each graph (black filled histogram). Histograms in A-D are representative of N=3 subjects per group. Histograms in E&F are representative of N=4 subjects per group.

Figure S9



Isotype control for flow cytometry analysis of the ability of AAT to modulate neutrophil membrane TNF- α interaction.

The ability of exogenous TNF- α (10ng/2x10⁷ cells) to bind neutrophil membranes was examined by flow cytometry. Cells were untreated (green histogram) or incubated with exogenous TNF- α (purple histogram) or TNF- α in the presence of physiological concentrations of AAT (27.5 μ M) (yellow histogram). Relative mouse IgG isotype control antibody (mouse IgG₁) is illustrated (black filled histogram). Represented result is one of three experiments.